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*THE INFECTION OF SUB-CELLULAR ESCHERICHIA COLI, STRAIN B, WITH A DNA PREPARATION FROM T2 BACTERIOPHAGE**

By Dean Fraser, Henry R. Mahler, Austin L. Shug, and Charles A. Thomas, Jr.†

DEPARTMENTS OF BACTERIOLOGY AND CHEMISTRY, INDIANA UNIVERSITY, BLOOMINGTON

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The ability of deoxyribonucleic acid (DNA) to carry genetic information was first demonstrated for the pneumococcus-transforming principle.[1] Even after the facts of transformation had been well established for several years, it was regarded by many as an anomaly, or at best of very limited scope, until the demonstration by Hershey and Chase[2,3] that the DNA of T2 bacteriophage carries all the genetic information necessary for reproduction of this virus. It is obvious that a system whose genetics is as well developed as that of the T2 phage[3] would be ideal for chemical genetic studies and investigations of the mechanism of action of fundamental genetic material. This paper describes experiments which demonstrate infection of protoplasts of *Escherichia coli*, strain B, with a preparation of disrupted T2 bacteriophage. As will become apparent, we believe the infective agent to be some form of DNA; but for the purposes of this discussion and pending physical and chemical characterization, we shall designate it as "DNA preparation" or simply "DNA."

METHODS AND MATERIALS

In general, the bacteriophage techniques were those described by Adams.[4]

Protoplasts.—We have modified our previous procedure[5] by concentrating the *E. coli* from 2×10^8 to 5×10^9 cells per milliliter (instead of to 5×10^8) and adding more lysozyme (0.1 ml. of 1 mg/ml per milliliter of bacteria) and more Versene (0.2 ml. of 1 per cent per milliliter of bacteria). An excellent and convenient semiquantitative criterion for the degree of protoplasting is the lack of streaming birefringence of the spherical protoplasts compared to the starting rodlike *E. coli*.

DNA Preparation.—Three milliliters of concentrated, purified T2 bacteriophage[6] at 5×10^{11} viable T2/ml was diluted into 27 ml. of concentrated urea solution (8 *M* urea in 0.1 *M* saline adjusted to pH 8.2 with hydrochloric acid). The phage titer dropped from the starting 5×10^{10} to about 10^5/ml in 1 minute and to about 500 viable T2 in 3 minutes. After 1 hour at 37° C., the preparation was dialyzed overnight at 2° C. (Visking tubing) against 3 liters of 0.1 *M* saline, with stirring inside and outside the dialysis tube, and then against a second 3 liters of 0.1 *M* saline for about 4–6 hours. Micro-Kjeldahl analyses of the dialyzate showed that all diffusible nitrogenous material was removed by this procedure. All such preparations

of phage DNA were viscous and slightly opalescent and contained no viable phage when plated straight and after dilutions in the usual medium.

A Standard "DNA Infection" Experiment.—Virtually all the experiments were done by slight variants of the following technique (all solutions and media have been previously described[5]). The aerated *E. coli* were grown in 3XD to 2×10^8 cells/ml, centrifuged, and resuspended in D at 10^9 cells/ml (when the protocol required pre-infection with T2) or in 0.1 *M* tris. The cells were then washed (usually twice) with cold 0.1 *M* tris buffer. The culture was resuspended at 5×10^9 in protoplasting medium, and a part was protoplasted as described above. For DNA infection, 0.5 ml. of either this preparation or a 1 : 10 dilution of it in protoplasting medium was mixed at room temperature with 0.5 ml. of the above DNA preparation, either straight or diluted 1:10 in 0.1 *M* saline. (The cell controls consisted of a sample of the unprotoplasted suspension treated in a parallel fashion.) After 10 minutes, this adsorption mixture was plated and/or diluted further in B-BSA.

B-BSA.—The "broth" of previously reported experiments[5] has been modified in the present work by the addition of bovine serum albumin (BSA). Armour 30 per cent sterile solution of BSA was diluted, with sterile technique, 1 volume into 14 volumes of sterile broth. This medium (B-BSA) was used for dilutions throughout the present experiments except as noted.

EXPERIMENTS AND RESULTS

Table 1 shows a typical experiment illustrating the infection of protoplasts with T2 "DNA." The chief feature of this experiment, as of all similar ones, is the complete absence of plaque-forming units in controls consisting of either protoplasts or "DNA" compared with the large numbers found in the mixture immediately, and with the greater numbers (some hundred fold) found after incubation of the mixture at 37° C. These experiments are completely reproducible and have been done many times with several lots of T2 "DNA." Because of the importance of establishing the nature of the infective agent, the remainder of this paper describes experiments designed to eliminate sources of misinterpretation and artifact and to establish the phenomenon beyond reasonable doubt.

Ordinary Controls—In all the experiments presented, blank platings of the plating bacteria were negative. Blank platings of the "DNA" and of experimental bacteria and protoplasts were made routinely both at the beginning of an experiment and after dilution into the nutrient medium (B-BSA) and incubation for the full time of the experiment. No experiment was considered valid unless the possibility of accidental pre-infection was completely eliminated by such negative controls.

TABLE 1*

INFECTION OF *E. Coli* PROTOPLASTS WITH T2 "DNA"

	Plaque Counts at Zero Time	Plaque Counts after 75 minutes at 37° C.
0.5 ml. T2 "DNA" + 0.5 ml. protoplasting medium	0	0
0.5 ml. T2 "DNA" + 0.5 ml. 1/10 protoplasts	6.5×10^5	6×10^7
0.5 ml. 0.1 *M* saline + 0.5 ml. 1/10 protoplasts	0	0

* In this and all similar experiments several dilutions in B-BSA were incubated and plated. The figures given are corrected for dilution to show plaque-forming units per ml. of original mixture.

"Masked" T2.—In no experiment was the total yield of recovered T2 more than a very small proportion (*ca.* 0.1 per cent) of the total T2 equivalents represented by the "DNA" used. In Table 1, for example, the input "DNA" was equivalent (in the adsorption tube) to 2.5×10^{10} T2/ml. The yield was 6×10^{7} T2/ml on the same basis. The following experiments were designed to eliminate the possibilities that (*a*) the DNA preparation contained T2 masked by the DNA, other degradation products, or traces of urea; (*b*) the "yield" represented initially inactive, but still intact, phage particles revivified in some way; or (*c*) the "yield" represented phage particles dissociated by the urea—i.e., into protein and DNA—and reconstituted by the action of protoplasts.

a) Possible inhibition of T2: Platings of several concentrations of T2 mixed with either 0.1 *M* or 0.01 *M* urea did not differ from control platings. Traces of urea in the preparation, therefore, are not inhibitory. An actual DNA preparation was also shown not to inhibit the plating of deliberately added T2.

b) Possible revivification of reversibly inhibited T2: Control platings of the DNA preparation diluted and incubated parallel to the experimental samples were consistently negative. The possibility of revivification by action of some agent leaking from the protoplasts—conceivably urease—was examined particularly because, by this hypothesis, protoplasts might be expected to show (as they did) much greater effects than intact cells. Logically, maximum effects would then be expected by exposure of the DNA preparation to a lysate. Tests were made with a lysate made by the identical treatment used for protoplasting, but with omission of sucrose from the protoplasting medium,[7] and also with sonically oscillated *E. coli*. Neither caused the appearance of T2 immediately or after incubation for 1 hour. The mixture of a standard experiment after adsorption of "DNA" and protoplasts showed 3,909 plaque-forming units/ml. When it was centrifuged cold for 12 minutes at 11,000 rpm in the Spinco No. 42 head, 100 per cent of the plaque-forming material sedimented, demonstrating that it was not free T2. Moreover, when the mixture of DNA and protoplasts was shocked into water at the end of the adsorption time, infective centers were completely eliminated. We have shown that identical treatment has no appreciable effect on ordinary T2.

Table 2 summarizes an experiment in which it was shown that the DNA preparation can infect protoplasts of T2-resistant B/2. The mechanism of "DNA infection" must, therefore, be quite different from the normal mechanism of cell inva-

TABLE 2*

INFECTION OF PROTOPLASTS OF *E. Coli* B/2 WITH T2 "DNA"

	Plaque Counts at Zero Time	Plaque Counts after 120 minutes at 37° C.
0.5 ml. B/2 protoplasts + 0.5 ml. 0.1 *M* saline	0	0
0.5 ml. B/2 protoplasts + 0.5 ml. T2 at 8.7×10^{5}/ml	8.6×10^{5}	9×10^{5}
Plated on B/2	0	0
0.5 ml. B/2 protoplasts + 0.5 ml. T2 "DNA"	800	2.7×10^{5}
Plated on B/2	0	0
Shocked 1/10 in water	0	0
0.5 ml. protoplasting medium + 0.5 ml. T2 "DNA"	0	0

* See footnote to Table 1.

sion by whole phage.[8] Since it is apparent that many of the possible complications of casual infection, etc., can be eliminated by the use of B/2, it might seem more sensible to have used this strain exclusively. This was not done for purely practical reasons, since B/2 grows much more slowly than B, is generally less convenient to handle, and, by our procedure, forms protoplasts somewhat less satisfactorily than B.

Any explanation of the present results which presupposes that the DNA preparation contained some form of T2 must assume further that the protoplast preparation contained some whole cells or else that protoplasts can be infected by ordinary T2. We have demonstrated repeatedly[5,7] that our preparations contain less than 0.1 per cent of viable cells by ordinary plating, and by direct microscopic observation we have found routinely less than 0.1 per cent (the limit of practical observation) of cell-like structures. We have failed completely in many attempts to infect protoplasts with intact T2 under conditions like those of the "DNA" experiments. Noninfection of protoplasts has, moreover, been the consistent finding of other workers in the field.[9]

c) Possible reconstitution of T2: The assumption might be made that DNA is being recoated with existing protein as in the reconstitution experiments of Fraenkel-Conrat[10] on tobacco mosaic virus. The protein might conceivably be present in a urea-denatured form re-natured by the action of protoplasts. Another assumption might be that DNA is entering the protoplast and synthesizing T2 protein coats with no net DNA synthesis. These assumptions are certainly possible in view of the low yield of phage compared to the total DNA input, but they require that the action occur either in the solution (a possibility which is eliminated by the centrifugation experiment above) or in the protoplasts themselves. In either case one would expect behavior quite different from that observed in the single-burst experiment of Table 3. By the former assumption, one would expect the action to be strongly cur-

TABLE 3*

SINGLE-BURST EXPERIMENT WITH PROTOPLASTS AND T2 "DNA"

	Plaque Counts at Zero Time	Plaque Counts after 3 Hours at 37° C.	Average Burst
1.5 ml. protoplasts + 1.5 ml. T2 "DNA"	6.9×10^4†	1.2×10^7†	180
1st 1/10 dilution	...	All samples *ca.* 1.2×10^7†	...
2nd 1/10 dilution‡	...	9, 181, 125, 31, 253, 127, 678 244, 399, 599, 366; 5 samples 0	247
3rd 1/10 dilution	...	110, 292, 227, 356; 12 samples 0	246

* The adsorption mixture was diluted 1/50 × 1/10 × 1/10 × 1/10 × 1/10, and one drop (0.069 ml.) of each of the 1/10 dilutions was diluted into each of 16 tubes (containing 1 ml. of B-BSA) which were incubated for 3 hours, mixed with 1 ml. of double-strength top agar containing bacteria, and poured on a plate.

† Corrected for dilution to original mixture.

‡ By Poisson distribution 3 tubes would be doubly infected.

tailed by dilution. If the reconstitution is occurring *within* certain protoplasts, we would predict from the fact that protoplasts are not limiting and from the one-hit nature of the data of Table 4 that, with rare exceptions, only a single phage could arise from each protoplast. As can be seen in Table 3, the average of the individual bursts which occur equals the burst size in mass culture.

Any assumption of co-operation between degradation products in a reconstitution requires a very strong dependence on the dilution of the "DNA." Table 4 shows

however, that the yield of infective centers is linear with dilution of the "DNA." This agrees with the experiments of Levinthal and Thomas,[11] which show that 40 per cent of T2 nucleic acid consists of a single "big piece" of DNA necessary for reproduction. The remaining 60 per cent is composed of smaller, apparently non-essential pieces. Since the "big piece" and the smaller pieces have been shown by star-counting to be separated by the urea treatment used in our experiments[12] and since the results of Table 4 preclude any co-operative action of more than one particle in infection, we concur in the belief that there is a single essential piece of DNA.

TABLE 4

DEMONSTRATION OF SINGLE-PARTICLE INFECTION BY T2 "DNA"

0.5 ml. Protoplasts + 0.5 ml. of:	Plaque Counts at Zero Time	Plating Dilution	Average Corrected for DNA Dilution
T2 "DNA" straight	32, 38	1/500	17,500
T2 "DNA" diluted 1/3	683, 466	1/10	17,250
T2 "DNA" diluted 1/9	259, 158	1/10	18,750
T2 "DNA" diluted 1/27	88, 85	1/10	23,600
T2 "DNA" diluted 1/81	32, 20	1/10	21,100
T2 "DNA" diluted 1/243	8, 7	1/10	18,200

The Infected Host.—In addition to the above experiments demonstrating that the early-eclipse infective centers are *not* T2, experiments have been done to show that they *are* all infected protoplasts. An odd feature of all the present "DNA infection" experiments was the immediate appearance of large numbers of infective centers. The experiments with pre-infected protoplasts indicated that they are unstable and that the eclipse period is characterized by essentially zero infective centers which survive plating.[7,13] This would seem to indicate that the DNA-infected host is not a protoplast.

The essence of the discrepancy between these and our previous results lies in the use of 2 per cent BSA in all dilutions of the present series of experiments. By direct microscopic observation and by turbidity measurements we have found, in accordance with Zinder and Arndt,[14] that protoplasts, either infected or uninfected, are tremendously more stable in a dilute BSA solution. We have now shown directly in experiments with pre-infected protoplasts (Fig. 1) that the BSA greatly enhances the apparent numbers of early infective centers. In the "DNA infection" we find quite similarly a few stable infected hosts but many more which survive plating after the addition of BSA. It is of interest to compare this finding with the parallel increase in the activity of pneumococcus-transforming DNA through the use of BSA.[15]

Figure 1 shows a single-step growth experiment in which we have compared the properties of pre-infected cells and pre-infected protoplasts with those of "DNA-infected" protoplasts. It is obvious that the infected host in the "DNA" experiment is like a protoplast in properties and not at all like an intact cell normally infected. It is interesting to note that in the two protoplast systems phage production is apparently not sharply terminated, i.e., the normal lysis mechanism is not operating.

Still further demonstration that the zero-time infective centers are actually infected protoplasts, as differentiated from stray infected cells and from stray T2, is the repeated observation that a 1-to-10 dilution through distilled water completely destroys the plaque-forming ability of the "DNA"-protoplast mixture (Table 2).

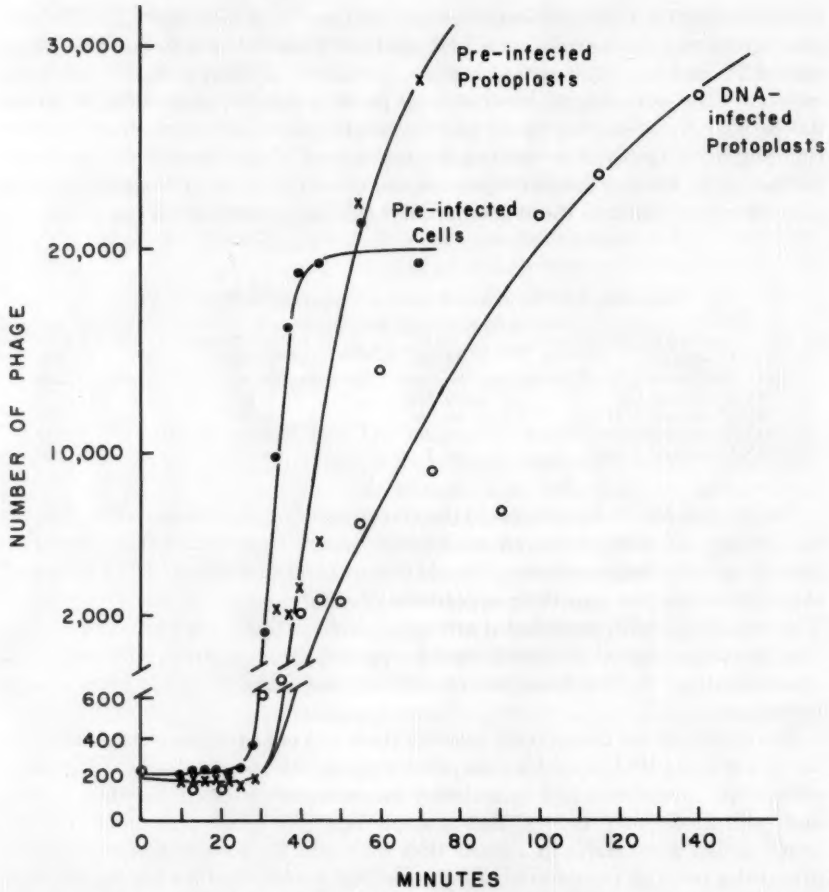

FIG. 1.—Comparison of single-step growth curves of pre-infected cells, pre-infected protoplasts, and "DNA-infected" protoplasts. Procedures described in the text. Latent-period plate counts arbitrarily adjusted to the same level. Considering dilution differences, this amounts to multiplying the pre-infected counts by 3, the "DNA-infected" counts by 2000.

The sum of our experience thus leads us to the belief that the host in "DNA infection" is certainly subcellular and probably the same protoplasts observed in our pre-infection experiments.[7] It is important to realize, however, that the objects of infection, at present efficiencies, might easily be a type far too rare to be observable.

The Nature of the Infecting Material.—Obviously it is essential to show not only that the infecting material cannot be T2 but that it *is* DNA. The most direct evidence at present is treatment of the urea preparation with DNase. One-half of a mixture of 3.1 ml. of T2 "DNA" and 3.1 ml. of water (total DNA estimated to be 150 μg.) was treated for 80 minutes at 37° C. with 0.1 ml. of 0.05 *M* $MgSO_4$ and 0.04 ml. of 1 mg./ml. DNase solution. The increase in optical density at 260 mμ in control experiments indicated essentially complete reaction in 5 minutes. Mixture

of such treated T2 "DNA" with protoplasts under standard conditions gave only some 3 per cent as many initial plaques as did the corresponding untreated half of the DNA material (3×10^4 versus 9.9×10^5). The remote possibility that the infecting material is T2 whose tail specificity has been altered by urea and whose DNA is now susceptible to DNase action cannot be discarded at present. The present results also do not exclude the possibility of protein bound to the DNA but show only that the T2 DNA is now accessible to DNase action, which was not true before urea treatment.

The Infection of "Whole Cells" with "DNA."—In virtually all the experiments on "DNA infection," controls have been run with non-protoplasted cells handled identically except for the addition of lysozyme and Versene. It was quite puzzling at first to find a small but persistent amount of infection in this control. This was, in fact, one of the main reasons for the great care which was taken to establish the absence of T2 in the preparation and the fact that the infected system behaves like protoplasts and not like cells. In the face of many completely negative control platings of cells and of the urea preparation alone and under experimental conditions identical except for the presence of the cells, we have been forced to the belief that it is indeed possible to infect non-protoplasted cells, particularly non-protoplasted B/2. The facts that this infection is reduced markedly by the prior action of DNase on the urea preparation and that it occurs with B/2 differentiate it sharply from normal T2 infection.

We invariably observed infection with the DNA preparation in cells which had been prepared for protoplasting, i.e., chilled, washed, and resuspended in the protoplasting medium before the addition of the DNA preparation. It is of interest to compare this with the enhanced cellular permeability effects observed by Jerne and Maaløe,[16] who found strong inhibitory action by RNase on the reproduction of T4 in *E. coli* suspended in distilled water. In previous experiments,[17] which will be reported separately, we have found that our washed cells always behave in part like protoplasts in that, although still rod-shaped, they are somewhat susceptible to osmotic shock, showing a loss of turbidity of about 25 per cent. Brief teatment of our pre-infected cells (i.e., ordinary whole cells) with RNase did not inhibit T2 growth,[5] but RNase treatment before "DNA infection" sharply inhibited phage reproduction, indicating to us that the particular "DNA" infectible cells are unusual and protoplast-like.

We suspect that this phenomenon has not been previously observed, primarily because it would not be detectable unless the T2 background were zero, as in our current preparation, and unless the cells had been made susceptible in some way. Because of the obvious importance of establishing whether such a DNA infection, possibly akin to pneumococcus transformation, might exist in nature, we intend to investigate this effect further.

DISCUSSION

Protoplasts, by our method and definition, are spherical bodies about the size of the original cell but much less stable to rapid changes in tonicity and incapable of forming colonies when plated on ordinary agar. We have also shown that under appropriate conditions our protoplasts are permeable to ribonuclease (RNase) and apparently to deoxyribonuclease (DNase), since both these enzymes lead to specific

removal of the homologous nucleic acid from protoplasts[12] and have strong effects on the reproduction of bacteriophage in protoplasts pre-infected with T2 and T3.[5] We have also found that specific antiphage serum can profoundly influence the course of phage reproduction in pre-infected subcellular *E. coli*.[18]

The infection with "DNA," then, is presumably another demonstration of the ready accessibility of the phage-replicating centers in protoplasts. In view of the tremendous size of the T2 DNA "big piece,"[11] one must question whether simple "permeability" is the answer. As alternatives, one might pose some specific entry mechanism or the hypothesis that the synthetic machinery is totally exposed.

The obvious items of next importance seem to us to include the separation, purification, and fractionation of the DNA and the improvement of the efficiency of infection to the point that genetic recombination experiments become possible. There would also seem little reason to suppose that the DNA from T2 is the only nucleic acid capable of instituting self-replication in *E. coli*. It will certainly be necessary to try using DNA from other viruses, DNA from genetically marked strains of bacteria, and even RNA with recognizable properties.

SUMMARY

T2 bacteriophage treated with 8 *M* urea is known to release much of its DNA. We have found that such a preparation was devoid of viable phage but, upon mixture with protoplasts of *E. coli*. strain B, and also the T2-resistant strain B/2, produced T2. Evidence has been presented to support the contention that actual infection of the protoplasts with DNA has occurred.

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Shortly after the preparation of this manuscript a paper appeared describing very similar observations (J. Spizizen, these PROCEEDINGS, **43**, 694, 1957). The methods of preparation of the protoplasts and of disrupted phage are quite different from those of our work, and the sensitivity of the infecting agent to various treatments (DNase, heat, Versene) appears to be at marked variance with our findings.

† Visiting from the Department of Physics, University of Michigan, Ann Arbor. Present address: Department of Biophysics, Johns Hopkins University, Baltimore, Maryland.

[1] R. D. Hotchkiss, *J. Cellular Comp. Physiol.*, **45**, (Suppl. 2), 1, 1955.
[2] A. D. Hershey and M. Chase, *J. Gen. Physiol.*, **36**, 39, 1952.
[3] A. D. Hershey, *Advances in Virus Research*, **4** (New York: Academic Press, Inc., 1957), 25.
[4] M. H. Adams, *Methods in Medical Research*, **2** (Chicago: Year Book Publishers, 1950), 1.
[5] D. Fraser and H. R. Mahler, *Arch. Biochem. and Biophys.*, **69**, 166, 1957.
[6] R. C. Williams and D. Fraser, *Virology*, **2**, 289, 1956.
[7] H. R. Mahler and D. Fraser, *Biochim. et Biophys. Acta*, **22**, 197, 1956.
[8] S. E. Luria, *General Virology* (New York: John Wiley & Sons, Inc., 1953), p. 164.
[9] M. R. J. Salton, *Bacteriol. Revs.*, **21**, 82, 1957.
[10] H. Fraenkel-Conrat and R. C. Williams, these PROCEEDINGS, **41**, 690, 1955.
[11] C. Levinthal and C. A. Thomas, Jr., *Biochim. et Biophys. Acta*, **23**, 453, 1957.
[12] Unpublished observations.
[13] M. R. Salton and K. McQuillen, *Biochim. et Biophys. Acta*, **17**, 465, 1955.
[14] N. D. Zinder and W. F. Arndt, these PROCEEDINGS, **42**, 586, 1956.

[16] M. S. Fox and R. D. Hotchkiss, *Nature*, **179**, 1322, 1957.
[16] N. K. Jerne and O. Maaløe, *Acta Pathol. et Microbiol. Scand.*, **40**, 362, 1957.
[17] Joan M. Sams, thesis, Indiana University, 1957.
[18] Janet Rowe, thesis, Indiana University, 1957.

*GENETIC INTERACTION AMONG STREPTOMYCETES: HETEROKARYOSIS AND SYNKARYOSIS**

BY DONALD H. BRAENDLE AND WACLAW SZYBALSKI

INSTITUTE OF MICROBIOLOGY, RUTGERS, STATE UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

Communication by Selman A. Waksman, September 13, 1957

The streptomycetes comprise a group of coenocytic filamentous microörganisms having many biological properties in common with the true bacteria, although superficially resembling the asexual molds. The industrial importance of certain species has focused considerable attention on the group as a whole. A wealth of information is available relating to their taxonomy, morphology, biochemistry, and other traits,[1] but, at the time the present work was begun, no experimental evidence for the existence of a sexual cycle within this group was available. The purpose of these studies was to survey the genus *Streptomyces* from the aspect of genetic interactions, to throw some light on the genetic mechanisms operating within these microörganisms, and to develop methods for controlled breeding experiments. Standard techniques for recombination analysis[2] in bacteria and for the study of heterokaryosis and sexual or parasexual reproduction in filamentous fungi were modified and applied to the streptomycetes. There were two main approaches: genetic analysis employing biochemically marked mutant strains, and cytological examination. The property of vegetative reproduction by the formation of uninucleate conidia proved to be useful.

Cytological Studies.—A colony of streptomyces is composed of three major elements: vegetative mycelium, aerial mycelium, and conidia formed in chains at the tips of the aerial hyphae. The nuclear structure of these elements was studied by light microscopy, utilizing the thionin stain with modifications described by Hunter-Szybalska *et al.*[3] Conidia of all the streptomyces species employed in this study appeared to be uninuclear, a very convenient feature for genetic studies. These nuclei divided after the conidia had been incubated approximately 3 hours in nutrient broth at 28° C. Germination with the production of one to two germination tubes occurred as early as 3 hours (*S. griseus*) to as late as 6 hours (*S. fradiae*), with still greater variation for several deficient mutants. The resulting vegetative mycelium was of very small diameter, varying from 0.6 to 0.8 μ, and was filled with an average of 8 chromatinic bodies for every 10 μ of length. The aerial mycelium was of approximately double thickness and exhibited a more distinct nuclear structure. No figures indicative of hyphal fusion and exchange of nuclei were observed with the species employed, in accordance with the genetic data, which imply a low frequency of heterokaryon formation. Visual observation of anastomosis was recently described by Gregory,[4] and the nuclear cytology of *Streptomyces* sp. T-12 has been discussed by McGregor.[5]

Isolation of Mutant Strains.—The procedure for the isolation of nutritionally deficient mutants comprised several steps. A homogeneous, filtered suspension of conidia in saline was irradiated with ultraviolet light at a dose permitting 1–10 per cent survival. This procedure increased the percentage of some types of mutants but was not always helpful.

The purpose of the next step was to increase the proportion of mutants in the irradiated suspension. The penicillin technique,[6] widely employed with bacteria, could not be successfully applied to streptomycetes. Several other antibiotics, including tyrocidin, novobiocin, catenulin, and mycomycetin, were also unsatisfactory, in that they either had no conidiocidal activity or failed to show any selective killing of germinating conidia. The filamentous character of streptomycetes suggested application of the filtration method, which was originally developed by Fries[7] for the fungus *Ophiostoma*. This method takes advantage of the fact that conidia which are allowed to germinate and produce mycelial growth will not pass through filter paper. After overnight incubation of a streptomyces conidial suspension in unsupplemented minimal broth,[8] there was over 90 per cent germination and mycelium formation by the prototrophic component, while virtually all the auxotrophic conidia remained unchanged. Subsequent filtration through sterile Whatman No. 2 filter paper resulted in a ten to fifty fold increase in the proportion of mutants. A very desirable feature of this method was the elimination of most of the partially blocked or "leaky" mutants, which germinate

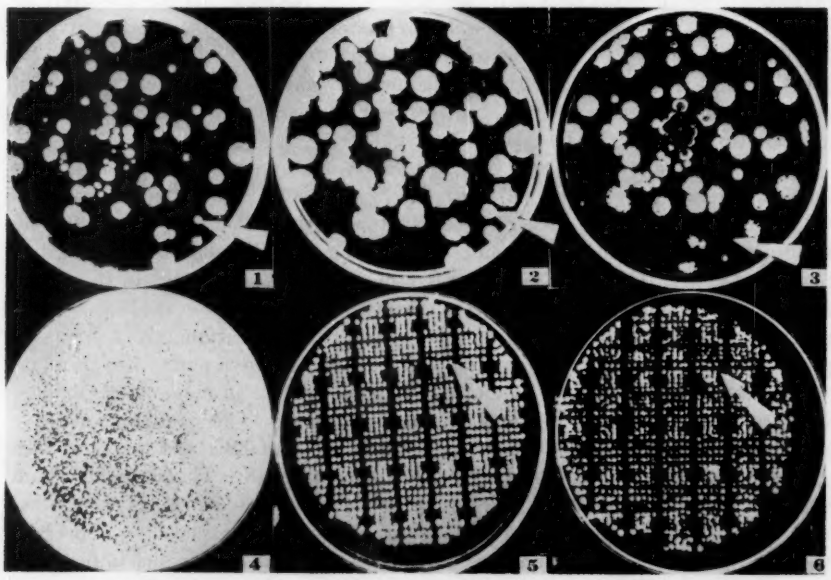

FIG. 1.—Isolation of auxotrophic mutants by means of replica plating employing velveteen (see n. 9) (dishes 1, 2, 3) or multiple-pin stamp (see n. 10) (dishes 4, 5, 6). The auxotrophic colonies grew on nutrient agar (shown by the arrows on dishes 2 and 5), but not on minimal agar (dishes 3 and 6). Dishes 1 and 4 represent the corresponding master plates.

even in unsupplemented minimal medium, forming a small amount of mycelium before growth ceases. This latter type of mutant was frequently encountered before the filtration step was introduced into the isolation procedure.

After filtration, the recovery of auxotrophic mutants was accomplished either by the replica-plating technique employing velveteen pads[9] or its modification by Szybalski,[10] which utilizes a stamp fitted with 300–2,000 needles in an orderly arrangement.[11] The latter method has two main advantages: more colonies can be transferred per plate without overlapping and smearing, and the identification of a particular mutant colony is facilitated by the orderly arrangement of the colonies (Fig. 1). Mutants were purified and identified by the auxanographic technique.

The procedure for the isolation of strains with multiple deficiencies was very similar to that described above. For the development of antibiotic-resistant mutants, the gradient-plate technique was employed.[12] Instability of marked strains stored at 2°C. on nutrient agar slants was occasionally encountered. For this reason, once or twice a year, strains were tested, purified, and lyophilized. The stability of lyophilized strains has not yet been determined on a large scale.

Nutritionally marked strains represented the following species: *S. albus* (2 mutants with single nutritional requirements), *S. coelicolor* R & M[18] (2 double), *S. coelicolor* NI 9021[18] (5 single), *S. fradiae* 3535 (5 single, 27 double), *S. griseus* 3475 (8 single, 10 double), *S. griseoflavus*[20] (3 double), *S. griseus* 3586 (11 single, 9 double), *S. parvus* 3686 (4 single), *S. sphaeroides* (3 single), and *S. venezuelae* 3625 (5 single). Additional markers included antibiotic production, pigmentation, and resistance to carbomycin, catenulin, cinnamycin, mycomycetin, novobiocin, streptomycin, tetracycline, tyrocidin, and viomycin. Several mutants exhibited alternative requirements, reflecting genetic blocks in biosynthetic pathways analogous to those found in other microörganisms. Some mutants, however, exhibited unusual alternative deficiencies, exemplified by the growth requirement of strain 4B23, a derivative of *S. griseus* 3475, which was satisfied by either of four amino acids: threonine, glycine, serine, or proline.

Demonstration of Heterokaryosis.—Exploratory experiments were performed on two mutants of the same strain of *S. griseus* (No. 3475) by a procedure similar to

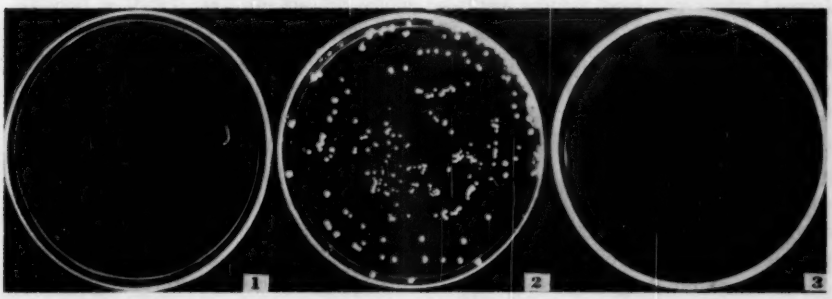

FIG. 2.—Formation of heterokaryotic colonies of *S. fradiae* on minimal agar. Dishes 1 and 3 were seeded with 10^8 conidia of strains 6F4-1 (met$^-$, isl$^-$, SM^S) and 6FS-16 (his$^-$, arg$^-$, SM^R), respectively. Dish 2, which received a mixture of parental conidia, shows the formation of prototrophic colonies.

that employed in the early crossing experiments with bacteria.[2] Conidial suspensions of mutants 4B24 and 4B26, histidine- and cysteine-deficient respectively, were spread on separate control plates of minimal agar and mixed together on a third similar plate, allowing a total of approximately 10^6 conidia per plate. After 4–6 days' incubation, 50–200 colonies appeared on the test plate inoculated with both mutants, while there was no growth on the control plates (Fig. 2). These colonies could be perpetuated on minimal agar only by massive conidial inoculation or by transfer of colonial fragments; single conidia grew only in the presence of the amino acids required by the parental stocks. These observations were consistent with the interpretation that the prototrophically growing colonies were of heterokaryotic origin, carrying both types of parental nuclei in the same mycelium.[13] More critical confirmation of heterokaryosis came from experiments in which small mycelial fragments, isolated by micromanipulation, were shown to give rise to prototrophically growing colonies. A test for syntrophy, performed by streaking heavy inocula of the parental strains in close proximity on minimal agar, was negative. The cumulative results of the above experiments justify the elimination of syntrophy as an alternative explanation for the emergence of prototrophic colonies. The absence of syntrophy and undesirable background growth with most of the mutants employed, as opposed to the pronounced syntrophy and thus higher yield of heterokaryons reported by Bradley and Lederberg,[13] reflects the use of the filtration technique in the isolation of obligate auxotrophic mutants.

Survey of Streptomycetes for the Occurrence of Heterokaryosis.—After heterokaryosis had been demonstrated between mutants derived from the same strain of *S. griseus*, strains of other species were put to the same test. Furthermore, heterokaryotic crosses were attempted between mutants derived from strains of diverse sources, belonging to the same or different species. Conidia participating in each cross were from two different sources: cultures of parental strains grown separately, and a mixed parental culture previously incubated for 10 days on a nutrient agar slant. Otherwise, the procedure was the same as in the preliminary experiments. As before, the inclusion of controls was designed to assess the rate of prototrophic reversion of the parental stocks and the contribution of syntrophy.

The results presented in Table 1 show three types of response. In the majority of the crosses between derivatives of the same strain, heterokaryotic colonies were formed when parental conidia came in close contact on the selective medium (minimal agar). In all these cases, the second test, which employed a conidial suspension derived from a mixed culture of the parents, also gave rise to the formation of prototrophic, heterokaryotic colonies, provided that the inoculum was at least 10^6 conidia per plate. In the case of *S. coelicolor*, no prototrophic colonies were formed directly from conidia on minimal agar, but inocula derived from mixed growth of the parents gave rise to a few stable prototrophic colonies. These were shown not to be of heterokaryotic nature and will be discussed in the following section on synkaryosis. Heterokaryotic growth of *S. coelicolor* mutants could be produced in the form of an outgrowth between two closely spaced parental colonies grown on minimal agar sparsely supplemented with nutrient broth (1.5 gm/liter). However, heterokaryons thus obtained could not be perpetuated on unsupplemented minimal agar. This behavior of the heterokaryotic mycelia of *S. coelicolor* indicates a defect in the mechanism of "cross-feeding" between parental nuclei in a

TABLE 1

Formation of Heterokaryotic Colonies

Parent I			Parent II			Growth on Minimal Medium	
Code	Derived from Strain	Markers*	Code	Derived from Strain	Markers*	Direct†	Secondary†
4B24		his$^-$	4B26		cys$^-$/met$^-$	+	+
4B24		his$^-$	4B22		cys$^-$/met$^-$	−	−
4B24		his$^-$	4B18		val$^-$ + isl$^-$	+	+
4B24		his$^-$	4B20		his$^-$	−	−
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B20	*S. griseus* 3475	his$^-$	+	+
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B18	*S. griseus* 3475	val$^-$ + isl$^-$	+	+
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B17	*S. griseus* 3475	arg$^-$	+	+
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B21	*S. griseus* 3475	his$^-$	+	+
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B24-5	*S. griseus* 3475	his$^-$; ser$^-$/gly$^-$	+	+
4B26	*S. griseus* 3475	cys$^-$/met$^-$	4B24-3	*S. griseus* 3475	his$^-$; ser$^-$/gly$^-$/thr$^-$	+	+
6F5		his$^-$	6F7		cys$^-$/met$^-$	+	+
6F5		his$^-$	6F4		met$^-$	+	+
6F5		his$^-$	6F2		arg$^-$/asp$^-$/glu$^-$	+	+
6F4		met$^-$	6F7		cys/met$^-$	+	+
6F4-1	*S. fradiae* 3535	met$^-$; isl$^-$/val$^-$	6F5-6	*S. fradiae* 3535	his$^-$; arg$^-$	+	+
6F4-1	*S. fradiae* 3535	met$^-$; isl$^-$/val$^-$	6F5-9	*S. fradiae* 3535	his$^-$; cys$^-$	+	+
6F4-2	*S. fradiae* 3535	met$^-$; arg$^-$/lys$^-$	6F5-7	*S. fradiae* 3535	his$^-$; val$^-$ + isl$^-$	+	+
6F4-2	*S. fradiae* 3535	met$^-$; arg$^-$/lys$^-$	6F5-9	*S. fradiae* 3535	his$^-$; cys$^-$	+	+
6F3-3	*S. fradiae* 3535	met$^-$; thr$^-$	6F5-16	*S. fradiae* 3535	his$^-$; arg$^-$, S^R	+	+
3E12	*S. albus* 618	met$^-$	3E13	*S. albus* 618	not detn.	+	+
2E1	*S. sphaeroides*	his$^-$	2E2	*S. sphaeroides*	met$^-$/cys$^-$	+	+
2C11	*S. parvus* 3686	arg$^-$	2C9-1	*S. parvus* 3686	cys$^-$/met$^-$; S^R	−	−
2C10	*S. parvus* 3686	his$^-$	2C11	*S. parvus* 3686	arg$^-$	−	−
14D1	*S. coelicolor* (R&M)	met$^-$; his$^-$	14D2	*S. coelicolor* (R&M)	pro$^-$; glu$^-$	−	+
14D1-2	*S. coelicolor* (R&M)	met$^-$; his$^-$; S^R	14D2	*S. coelicolor* (R&M)	pro$^-$; glu$^-$	−	+
13D3	*S. coelicolor* N.L. 9021	met$^-$	13D4	*S. coelicolor* N.L. 9021	arg$^-$	−	+
7A3	*S. venezuelae* 3625	tyr$^-$	7A26	*S. venezuelae* 3625	arg$^-$/asp$^-$/glu$^-$	−	−
7A4	*S. venezuelae* 3625	arg$^-$	7A32	*S. venezuelae* 3625	not detn.	+	+
7A4	*S. venezuelae* 3625	arg$^-$	7A26	*S. venezuelae* 3625	arg$^-$/asp$^-$/glu$^-$	−	−
1G1	*S. griseoflavus*	ade$^-$, met$^-$	1G2	*S. griseoflavus*	gly$^-$, try$^-$	+	+
14D2	*S. coelicolor* (R&M)	pro$^-$, glu$^-$	13D3	*S. coelicolor* N.L. 9021	met$^-$	−	+
3B64	*S. griseus* 3586	pro$^-$; leu$^-$	4B24-4	*S. griseus* 3475	his$^-$; met$^-$	−	−
3B66	*S. griseus* 3586	pro$^-$; thr$^-$	4B19-1	*S. griseus* 3475	arg$^-$; met$^-$	−	−
4B24	*S. griseus* 3475	his$^-$	14D1	*S. coelicolor* (R&M)	his$^-$; met$^-$	−	−
4B24	*S. griseus* 3475	his$^-$	7A4	*S. venezuelae* 3625	arg$^-$	−	−
4B24	*S. griseus* 3475	his$^-$	2C9	*S. parvus* 3686	cys$^-$/met$^-$	−	−
4B24	*S. griseus* 3475	his$^-$	6F7	*S. fradiae* 3535	cys$^-$/met$^-$	−	−
14D2	*S. coelicolor* (R&M)	pro$^-$; glu$^-$	7A4	*S. venezuelae* 3625	arg$^-$	−	−
3E12	*S. albus* 618	met$^-$	2E1	*S. sphaeroides*	his$^-$	−	−
3E13	*S. albus* 618	not detn.	2E2	*S. sphaeroides*	met$^-$/cys$^-$	−	−
1G2	*S. griseoflavus*	gly$^-$; try$^-$	14D2	*S. coelicolor* (R&M)	pro$^-$; glu$^-$	−	−

* Ade = adenine; arg = arginine; asp = aspartic acid; cys = cysteine; glu = glutamic acid; gly = glycine; his = histidine; isl = isoleucine; leu = leucine; lys = lysine; met = methionine; pro = proline; ser = serine; thr = threonine; try = tryptophane; tyr = tyrosine; val = valine; S^R or S^S = streptomycin-resistant or -sensitive.

† Colonies were scored on minimal agar (see n. 8) after plating approximately 10^8 conidia derived from separate slant cultures of Parents I and II (direct), or from a slant on which a mixed culture of the parents was grown for a period as long as 10 days (secondary). The plus sign denotes formation of a considerable number of colonies, while the corresponding controls for syntrophism or for reversions of the parental strains showed no growth or a negligible number of colonies.

common cytoplasm. "Nutritionally unbalanced" heterokaryosis was also occasionally observed in crosses between doubly deficient strains of *S. fradiae* on minimal medium supplemented with two out of four required amino acids, in combinations, which would not permit growth of any of the parents. Some of the heterokaryotic colonies obtained under such selective conditions could not be perpetuated on minimal agar, unless one or two of the amino acids previously used in the selective medium were present. However, they produced both unaltered types of parental conidia, in analogy with the "anomalous heterokaryons" described by Bradley and Lederberg.[13] Shifts in the nuclear ratios in such heterokaryotic mycelia might be responsible for this behavior.

Crosses between certain mutants derived from the same strain and all the crosses between mutants of diverse origin, whether or not the parental strains were classified in the same species, invariably were negative, i.e., no prototrophic, heterokaryotic colonies were produced.[15] Incompatibility between different strains might have important taxonomic significance and should be the subject of more inclusive studies. A system analogous to the SRP type of cross, employed by Lederberg[16] for the survey of sexual compatibility among *Escherichia coli* strains, would facilitate a broader survey of streptomyces strains for heterokaryotic compatibility. An attempt to develop such a system, however, showed the property of streptomycin resistance to be recessive in heterokaryotic mycelia; heterokaryons between streptomycin-resistant and -sensitive strains could not be formed or easily propagated on streptomycin-containing minimal agar. A search for a dominant resistance marker is under way.

Synkaryosis among Streptomycetes.—After the establishment of a heterokaryon, a more intimate association between the unlike parental nuclei in the same cytoplasm may result in occasional nuclear fusion and the formation of a more or less transient heterozygous diploid nucleus. This second type of genetic interaction, denoted "synkaryosis," was hitherto demonstrated only with *S. fradiae* and *S. coelicolor.*[14, 17, 20] The latter is the organism of choice for experimentation because of a reasonably high frequency of synkaryosis and because, with the mutants employed, no heterokaryotic colonies were formed from a mixture of parental conidia on selective, minimal agar. The test for synkaryosis was analogous to crossing experiments with asexual fungi.[2] Heterokaryotic growth was allowed to form between the parental strains on complete or selective media, and the resulting conidia were analyzed for their growth requirements. The detection of conidia which subsequently bred true and exhibited different combinations of non-parental markers was considered as indicative of synkaryosis, as opposed to heterokaryosis, where only parental types of conidia are produced.

In the crossing procedure for *S. coelicolor,*[18] a mixture of parental conidia was incubated on nutrient agar for 8 days, and then the harvested conidia were plated on minimal media supplemented with two out of the four amino acids required by the parents in combinations which would not permit growth of either parent. The resulting colonies proved to be stable recombinants; the results of a typical experiment are summarized in Table 2. Table 3 represents a similar experiment performed with mutants of *S. fradiae.* In the latter case the experimental procedure was necessarily modified to compensate for two complicating factors. Because of a lower frequency of synkaryotic occurrence, a massive growth of heterokaryotic

TABLE 2

NUMBER AND TYPES OF COLONIES RECOVERED AS PROGENIES OF CROSSES BETWEEN MUTANTS OF *S. coelicolor* (RECIPROCAL FOR STREPTOMYCIN RESISTANCE)

	Minimal Agar Medium Supplemented By																										Parental Strains	
	Met + Glu								His + Glu						His + Pro				Met + Pro									
Number of Colonies	12	6	1*	1*	8	7	3	2*	12	1	5	1*	2*		8	1	6		5	2	4	7	1	1*			15D1-2	14D2
Colony type: met	+	+	++	++	+	+	+	++	+	+	+	++	++		+	+	+		+	+	+	+	+	+−			−	+
his	+	+	+−	++	+	+	+	++	+	+	+	++	++		+	+	+		+	+	+	+	+	++			−	+
pro	+	+	++	++	+	+	+	++	+	+	+	+−	++		+	+	−		+	+	+	−	−	+−			+	−
glu	+	+	++	++	+	+	+	++	+	+	+	++	++		+	+	+		+	+	+	+	+	++			+	−
pigm	+	±†	+−	+−	+	−	±†	+−	+	−	±†	++	+−		+	−	−		+	−	±†	+	±†	++			+	+
SM	R	R	R S	R R	R	R	R	R	R	R	R	R R	R R		R	R	R		R	R	R	R	R	R R			R	S

Number of Colonies	11	2	6	1*	18	1	1		7	1	4	2	1*	1*	4	1	4	8	1	2	3	4	1	6	2*	3*	1*	14D2-1	14D1
Colony type: met	+	+	+	++	+	+	−		+	+	+	+	+−	++	+	+	+	+	+	+	+	+	−	−	−−	−−	−+	+	−
his	+	+	+	++	+	+	+		+	+	−	−	++	−+	+	+	+	−	−	+	+	+	+	+	+−	++	++	+	−
pro	+	+	+	++	+	+	+		+	+	+	+	−+	−+	+	+	+	+	+	−	+	−	+	−	−+	−+	++	−	+
glu	+	+	+	++	+	+	+		+	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	++	++	++	−	+
pigm	+	−	±†	+−	+	±†	+		+	−	+	−	+−	++	+	−	±†	+	−	+	+	+	+	−	−+	−+	++	−	+
SM	S	S	S	S S	S	S	S		S	S	S	S	R S	S S	S	S	S	S	S	S	S	S	S	S	R S	R R	R S	R	S

* Colony containing a mixture of approximately equal numbers of two or more types of conidia.
† Weak and late producton of pigment (probably could be classified as pigm⁻).

TABLE 3

NUMBER AND TYPES OF COLONIES RECOVERED AS PROGENIES OF CROSSES BETWEEN MUTANTS OF *S. fradiae* (RECIPROCAL FOR STREPTOMYCIN RESISTANCE)

	Minimal Agar Medium Supplemented By																											Parental Strains	
	Met + His								Met + Arg				Isl + His								Isl + Arg								
Number of Colonies	48				2	30	16	1	47	2			2	13	1*	1*	3*	29*	1*		27	21	1*					6F4-1	6F5-16
Colony type: his	+				+	+	−	−	+	+			+	−	+−	−−	++	+−	+−		+	+	++					+	−
arg	+				+	+	+	+	+	−			+	+	++	++	++	++	+−		+	−	+−					+	−
met	+				+	−	+	−	+	+			+	+	++	+−	−+	−+	−+		+	+	++					−	+
isl	+				+	+	+	+	+	+			+	+	++	++	++	++	++		+	+	++					−	+
SM	R				R	S	R	R	R	R			R	R	R R	R R	S R	S R	S R		R	R	R R					S	R
Class†	R_p				R_p	i	a	R_n	R_p	h			R_p	a	R_p a	a R_n	i R_p	i a	i a		R_p	h	R_p h					p	p

Number of Colonies	50		3*		13	27	1*	1*	10	17	22	1*	48	1	1*						6	2	19*	5*	10*			6F4-10	6F5-13
Colony type: his	+	+	−	+	+	+	−+	++	+	+	+	++	+	−	+−						+	+	++	+−	++			+	−
arg	+	+	−	+	+	+	−+	++	+	+	−	+−	+	+	++						+	−	+−	+−	++			+	−
met	+	+	+	−	+	−	−+	−+	+	−	+	++	+	+	++						+	+	−+	−+	−+			−	+
isl	+	+	+	−	+	+	++	++	+	+	+	++	+	+	−+						+	+	++	++	++			−	+
SM	S				S	R		S S	S	R	S	S S	S	S	R S						S	S	R S	R S	R S			R	S
Class†	R_p	R_p	p	p	R_p	i	$R_n R_p$	$R_n R_p$	R_p	i	h	R_p h	R_p	a	m a						R_p	h	i h	i p	i R_p			p	p

* Cf. Table 2.
† R_p = prototrophic recombinant; R_n = non-prototrophic recombinant; p = parental; h, a, m, i = probable revertants at his, arg, met, isl/leu loci, respectively.

mycelia was maintained on a selective medium for a period of 10 or more days. The tendency of *S. fradiae* mutants to form secondary prototrophic heterokaryons on selective media necessitated introducing an additional step into the procedure for detecting recombinants. Conidia harvested from the primary heterokaryon were plated on supplemented minimal media, permitting the recombinants to form colonies and to *sporulate*. The frequency of secondary heterokaryon formation was low enough that the proportion of recombinant conidia in the total harvest was greatly increased by this step. In a second plating on selective media, interference by secondary heterokaryons was minimized by adequate dilution of the conidial suspension, and numbers of stable haploid recombinants were recovered. The experimental details of these studies have been presented elsewhere.[19]

Genetic analysis of synkaryotic interaction in both streptomyces species led to similar conclusions. A salient feature was the preferential formation of prototrophic recombinants in four-factor crosses on a variety of doubly supplemented minimal media (Tables 2 and 3). That these were not diploid could be concluded from the behavior of the streptomycin resistance marker, which, on the basis of of segregation data in non-prototrophic recombinants, seemed to be linked very strongly either to isoleucine (*S. fradiae*) or to glutamic acid (*S. coelicolor*), although evidence of the latter was not consistent. If the notion of persistent diploidy[21] were true, all the products of both reciprocal crosses would be expected to be streptomycin-sensitive, assuming that the streptomycin resistance marker behaves as a recessive character. The objection that the prototrophs may have originated from double reverse mutation of one parent may also be dismissed, because of the very low frequency of conidia which may have originated as products of a single reversion. Further indications as to the mechanism of synkaryosis came from the rare recovery of colonies which were derived from a single spore or filterable mycelial fragment, and which contained a mixture of conidia: two of parental type and two with non-parental combinations of markers.

The prevalence of prototrophic recombinants and the scarcity or lack of the complementary types of recombinants pointed to the intervention of strong selective forces in the period intervening between the occurrence of recombination and the scoring of the recombinant colonies. The non-prototrophic recombinant nuclei seemed either to multiply more slowly or to produce a lower yield of conidia, even on selectively enriched media. Until methods are devised for the recovery of all possible classes of recombinants resulting from a cross, any mapping of the relative position of characters on chromosomes of the employed strains would be premature, although the methods could be successfully applied to the production of several types of recombinants, some of which might prove useful for other theoretical or practical purposes.

Summary.—Heterokaryosis in streptomycetes denotes a co-operative venture in which genetically unlike nuclei perpetuate within a common cytoplasm as the result of hyphal anastomosis. Conidia formed by heterokaryons are of parental type only. Heterokaryosis, as defined, has been demonstrated between mutants of the same strain of *S. griseus*, *S. griseoflavus*, *S. fradiae*, *S. venezuelae*, *S. albus*, *S. sphaeroides*, and *S. coelicolor*, but not between mutants of diverse origin. Heterokaryons between amino acid-requiring mutants of the last species, and occasionally of other species were "nutritionally unbalanced," i.e., did not grow on minimal media unless at least a portion of the parental requirements was supplied.

Synkaryosis is the fusion of unlike nuclei within heterokaryons and subsequent reassortment of genetic characters to yield conidia of recombinant types. Two species, *S. coelicolor* and *S. fradiae*, have exhibited the synkaryotic type of interaction, although the quantitative results were strongly biased by apparent selective forces active during the period intervening between the actual karyogamy and the scoring of recombinant colonies.

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[1] S. A. Waksman, *The Actinomycetes*. Waltham, Mass.: Chronica Botanica Co., 1950.

[2] G. Pontecorvo, *Advances in Genet.*, **5**, 141–238, 1953; J. Lederberg, *Genetics*, **32**, 143–63, 1947.

[3] M. E. Hunter-Szybalska, W. Szybalski, and E. DeLamater, *J. Bacteriol.*, **71**, 17–24, 1956.

[4] K. F. Gregory, *Can. J. Microbiol.*, **2**, 649–55, 1956.

[5] J. F. McGregor, *J. Gen. Microbiol.*, **11**, 52–56, 1954.

[6] B. D. Davis, *J. Am. Chem. Soc.*, **70**, 4267, 1948; J. Lederberg and N. Zinder, *J. Am. Chem. Soc.*, **70**, 4267, 1948.

[7] N. Fries, *Nature*, **159**, 199, 1947.

[8] 7 gm. K_2HPO_4; 2 gm. KH_2PO_4; 0.25 gm. Na citrate$\cdot 5H_2O$; 0.1 gm. $MgSO_4 \cdot 7H_2O$; 1 gm. $(NH_4)_2SO_4$; 2 gm. glucose; the solid minimal medium contained 1.6 per cent thoroughly washed agar.

[9] J. Lederberg and E. M. Lederberg, *J. Bacteriol.*, **63**, 399–406, 1952.

[10] W. Szybalski, *Microbial Genet. Bull.*, No. 13, p. 42, 1956.

[11] "Flower frogs," 8 cm. in diameter, can be adapted for this purpose. They are commercially available through flower shops and are sterilized by dipping the needles in alcohol and flaming.

[12] W. Szybalski, *Science*, **116**, 46–48, 1952.

[13] W. Szybalski and D. H. Braendle, *Bacteriol. Proc.*, Vol. **48**, 1946; S. G. Bradley and J. Lederberg, *Bacteriol. Proc.*, Vol. **48**, 1956; *J. Bacteriol.*, **72**, 219–25, 1956.

[14] G. Sermonti and I. Spada-Sermonti, *J. Gen. Microbiol.*, **15**, 609–16, 1956.

[15] Bradley and Lederberg (Bacteriol. Proc., Vol. **48**, 1956) reported heterokaryon formation in interspecific crosses between fresh soil isolates of *S. griseus* and *S. cyaneus*.

[16] J. Lederberg, *Science*, **114**, 68–69, 1951.

[17] D. H. Braendle and W. Szybalski, *Bacteriol. Proc.*, Vol. **52**, 1957; W. Szybalski, in *Neomycin: Its Nature and Practical Application*, ed. S. A. Waksman (in press); and D. A. Hopwood, *J. Gen. Microbiol.*, **16**, ii–iii, 1957.

[18] The streptomycin-sensitive mutant strains of *S. coelicolor* R & M (14D1 and 14D2; cf. Table 1) were kindly supplied by Dr. G. Sermonti, of the Istituto Superiore di Sanità, Rome, Italy. We also wish to thank Dr. K. Tsubaki, of Nagao Institute, Tokyo, Japan, for the prototrophic strain NJ-9021 of *S. coelicolor*.

[19] D. H. Braendle, "Cytology and Genetics of Streptomyces" (Ph.D. thesis, Rutgers University, May, 1957), pp. 1–134.

[20] Heterokaryosis and possible karyogamy obtained with strains of *S. griseoflavus* were reported by H. Saito and Y. Ikeda at the annual meeting of the Society for Agricultural Chemistry of Japan, April 9, 1957. Heterokaryosis and synkaryosis were confirmed in our preliminary experiments with strains 1G1 and 1G2 kindly supplied by Dr. H. Saito, of the University of Tokyo.

[21] S. G. Bradley, *J. Bacteriol.*, **73**, 581–82, 1957.

THE ACTIVATION OF METABOLIC SYSTEMS DURING CROWN-GALL TUMOR-CELL FORMATION

By Richard M. Klein

NEW YORK BOTANICAL GARDEN, BRONX PARK, NEW YORK 58, NEW YORK

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INTRODUCTION

The transformation of normal plant tissue into tumor tissue occurs in three steps, which have been designated conditioning, induction, and promotion.[1] Conditioning is accomplished by substances present in the wound juice; induction by a tumor-inducing principle (T-iP) produced by virulent *Agrobacterium tumefaciens* grown in unheated plant juice; and promotion, by indoleacetic acid or its physiological equivalent. For carrot, complete conditioning requires 16–20 hours at 25° C.; induction, an additional 60–70 hours; and promotion, another 28–30 hours—a total of 104–120 hours is necessary for optimal transformation. Incomplete conditioning, induction, or promotion results in weakly growing tumors. If resulting tumor growth is submaximal because of the inability of the tumor cell to synthesize sufficient amounts of a necessary factor, supplements of the factor should increase growth. Rapidly growing tumors, receiving optimal transformation, should not be simulated by any growth factors. The experimental evaluation of this hypothesis[2] is reported in this paper.

MATERIALS AND METHODS

A carrot phloem disk bioassay[3] was used throughout this study. Disks of phloem were inoculated with *ca.* 10^6 bacteria from an 18–24-hour broth culture. All disks were incubated at 25° C. in the dark, except during manipulation and for temperature studies. At the end of the transformation period, the disks were transferred from moistened filter paper to Petri dishes containing 20 ml. of 0.5 per cent agar plus the compounds to be tested. This concentration of agar did not alter the final fresh or dry weights of tumor tissues as compared to filter-paper controls. After 15 days from 0 time (10 days after the end of transformation), the tumor tissue was removed from the carrot disks and weighed. Eight disks were used per variable, and all experiments were repeated at least once.

RESULTS

1. *Incomplete Conditioning.*—Two methods were employed to secure tumors whose growth was slow because of incomplete conditioning. In the production of tumor tissue on inoculation with virulent crown-gall bacteria, individual carrot roots could be divided into strong (SR), moderate (MR), and weak reactors (WR).[4] These differences are due to a lower supply of conditioning factors in the MR and WR carrots as compared to SR carrots. By selecting pretested carrots, disks possessing any desired tumor-forming capacity could be obtained. Incomplete conditioning was also accomplished by holding disks of SR carrots for 24 hours at 4°–6° C., which reduced subsequent tumor growth to about 50 per cent of that obtained at 25° C. Carrot disks which were incompletely conditioned but which subsequently received induction and promotion treatments adequate for maximum

tumor development were placed on agar to which various supplements were added, for a 10-day period following transformation. A 100-fold concentration range of 1,3-diphenylurea, naphthaleneacetic acid (NAA), gibberellic acid, pyrimidine ribosides, mevalonic acid, and a mixture of B vitamins in the proportions found in yeast extract had no effect on the growth of these tumors. "Vitamin free," acid-hydrolyzed casein, yeast extract, adenosine, guanosine, kinetin, and coconut milk each caused significant increases in the growth of slow-growing tumors which resulted from incomplete conditioning accomplished by either of the two methods used. Fully conditioned SR disks were not stimulated by any of the supplements tested. Adenosine and guanosine were equally effective. The positive results obtained with kinetin were due to its purine riboside structure and not to any special properties of the kinetin molecule. The failure of "B" vitamins to augment tumor-cell duplication suggests that yeast extract acted because of its amino acid content, a finding which may also apply to coconut milk. Casein hydrolyzate could be replaced by a mixture of nineteen amino acids.

If amino acids and purines were the only factors required for rapid multiplication of tumors which grow slowly because of incomplete conditioning, their simultaneous addition should have increased the growth of such tumors to that of fully conditioned SR tumors. Since this did not occur, the other systems may also be involved in the conditioning process.

2. *Incomplete Induction.*—Two techniques were used to obtain slow-growing tumors resulting from incomplete induction. The first was developed by Braun,[5] who found that the action of T-iP could be prevented by holding inoculated tissues for a portion of the induction phase at 32° C., a temperature at which T-iP either is inactivated or is not synthesized by virulent crown-gall bacteria. Inoculated disks of SR carrot phloem were held at 25° C. for 0, 24, or 48 hours (following a 24-hour period at 25° C. for full conditioning) and were then transferred to 33° C. for the balance of the induction phase. The other technique involved the use of strains of crown-gall bacteria which differ in virulence bedause of their genetic capacity to synthesize a quantitatively or qualitatively inadequate T-iP.[6]

Fully conditioned and fully promoted, but incompletely induced, disks were transferred to agar containing various supplements for 10 days following transformation. Casein hydrolyzate, purine or pyrimidine ribosides, yeast extract, 1,3-diphenylurea, and NAA were ineffective over a 100-fold range in concentration. Kinetin, gibberellic acid, mevalonic acid (β,δ-dihydroxy-β-methyl valeric acid) and coconut milk stimulated slow-growing tumors which had received 24 or 48 hours of T-iP action but were without effect on fully induced tumors or on tumors receiving only minimal induction. The same compounds were effective supplements for slow-growing tumors formed following inoculation with moderately or weakly virulent strains of crown-gall bacteria (Fig. 1) but were without effect on disks inoculated with avirulent bacteria (strain IIBNV6) or on tumors induced by highly virulent bacteria (strains B6).

3. *Incomplete Promotion.*—Incomplete promotion was accomplished by presenting an antiauxin to tissues during the promotion phase. Of several tested, 2,4-dichloroanisol proved most satisfactory, since its presentation during induction did not inhibit subsequent tumor growth, i.e., there was no carry-over of the antiauxin. During the promotion phase, 2,4-dichloroanisol in agar was supplied to fully con-

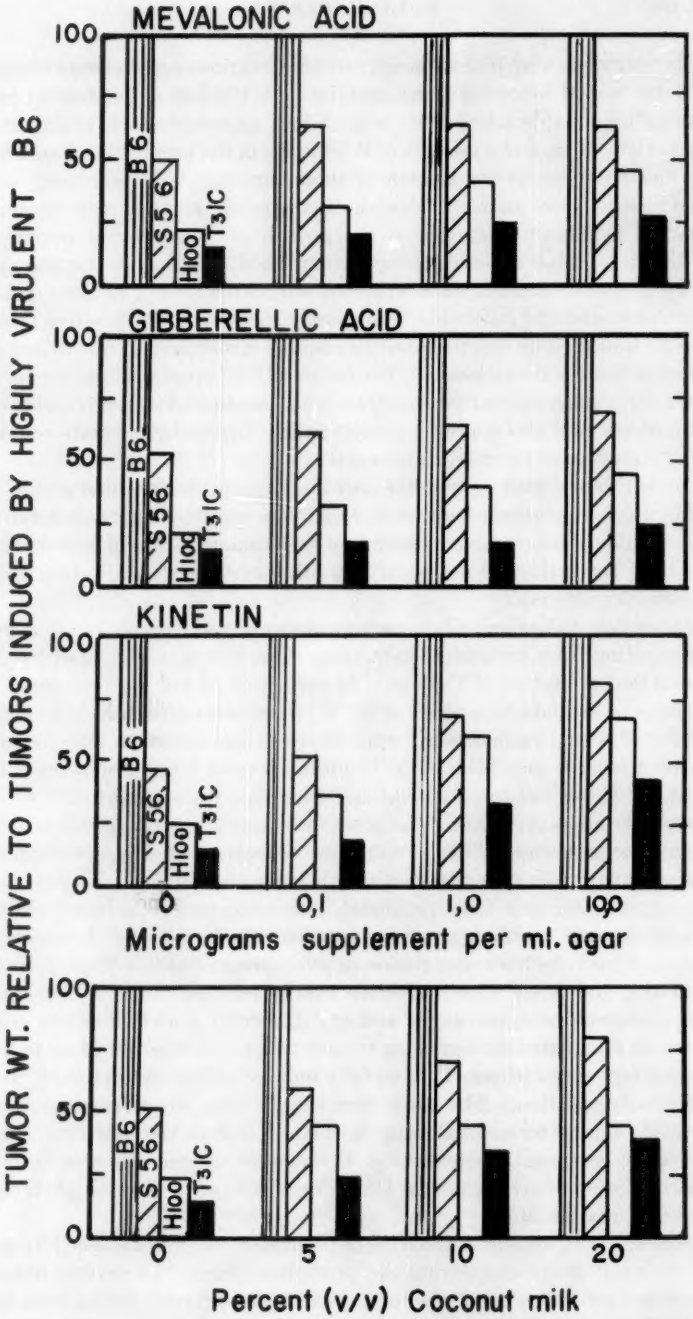

FIG. 1.—Effects of supplements on weakly active crown-gall tumors resulting from inoculation with strains of *A. tumefaciens* differing in virulence.

ditioned and induced SR disks. The potential 2,4-dichloroanisol inhibition of promotion and, hence, of subsequent tumorous growth was almost completely prevented by simultaneously supplied auxin. When the antiauxin was used alone during promotion, the resulting slow-growing, incompletely promoted tumor cells were stimulated by auxin applied during the growth period. To date, nonauxinic supplements have not been tested, but the completeness of the auxin effect suggests that other substances may not be involved.

DISCUSSION

Since slow-growing plant tumors resulting from incomplete conditioning, induction, or promotion are stimulated by furnishing them suitable supplements, these tumors probably suffer from metabolic deficiencies which are supplied by the supplements furnished. The possibility that the slower growth is caused by inhibitors in the tissue which are antagonized by the supplements cannot be eliminated, but the assumption of deficiencies appears more likely.

Through incomplete conditioning, induction, or promotion, three biochemical types of slow-growing tumors were produced, each characterized by its own set of nutritional deficiencies. Tumors resulting from incomplete conditioning required amino acids and purines. Kinetin, gibberellic acid, mevalonic acid, and coconut milk stimulated incompletely induced tumors, and only auxin stimulated incompletely prompted tumors. The rapidly growing tumors which developed when the tissue was subjected to full treatment in all three steps did not respond by increased growth to any of the tested substances. We interpret this to mean that the metabolic systems deficient in the weak tumors are fully active in the strong tumors, an interpretation in harmony with data on the composition of tumors.

It appears probable that tumor-cell transformation, at least for carrot tissue, involves the permanent establishment of metabolic systems which do not exist, or exist in minimal effectiveness, in nontumorous cells or the unmasking or activation of existing systems which, for some reason, are ineffective in nontumorous cells. These facts suggest that the etiological agents functioning during the different steps in tumor-cell formation directly or indirectly activate one or more metabolic or enzyme systems whose products are necessary for maximal expression of the growth potential of tumor cells. In several steps the known etiological agent is chemically related to the required product of the activated metabolic system. Permanent activation may occur by a feedback mechanism, wherein the metabolic system, once in operation, forms or does not destroy the compounds necessary to keep it functioning.[7] This differs from experimental induction of bacterial enzymes where the substrate induces the formation of an enzyme system destroying the substrate.

The effect of supplements in promoting the multiplication of tumor cells which are slow-growing because of incomplete transformation is temporary, not permanent. These substances supply a deficiency, but they do not induce the development in tumor cells of persistent metabolic systems which continue to supply the deficiency. They do not make weak tumors become permanently strong tumors. Braun[8] has suggested that the activated metabolic systems are "fixed" by agents acting during subsequent steps. It is also possible that, once cells have been transformed into tumor cells, their metabolic systems are insusceptible to the action of the etiological agents.

It must be emphasized that the ability of tumor cells to synthesize growth factors is not the entire "cause" of crown gall. Morphogenic autonomy of tumor cells is found after transformation periods shorter than those required for full activation of all these metabolic systems, and there is no evidence that certain respiratory and energy-metabolism characteristics of tumors[2] are related to the growth factors. Finally, the addition of the factors to unwounded carrot phloem tissues has not resulted in the appearance of neoplastic tissues. Braun[8] has, however, obtained self-limited neoplasms grossly similar to tumors when the sensitive, totipotent cells of tobacco pith were supplied with several growth factors.

Details of these experiments will be published elsewhere.

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[1] R. M. Klein and G. K. K. Link, *Quart. Rev. Biol.* **30,** 207–277, 1955.
[2] R. M. Klein, *15th Growth Symposium, Soc. Study, Devel. Growth* (1956).
[3] R. M. Klein and I. L. Tenenbaum, *Am. J. Botany,* **42,** 709–712, 1955.
[4] R. M. Klein, these PROCEEDINGS, **41,** 271–274, 1955.
[5] A. C. Braun and R. J. Mandle, *Growth,* **12,** 255–269, 1948.
[6] D. T. Klein and R. M. Klein, *J. Bact.,* **72,** 308–313, 1956.
[7] Vogel, H. J., these PROCEEDINGS, **43,** 491–496, 1957.
[8] A. C. Braun, *Decentennial Tissue Culture Conference* (1956).

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*INDUCTION OF BOLTING AND FLOWERING IN HYOSCYAMUS AND SAMOLUS BY A GIBBERELLIN-LIKE MATERIAL FROM A SEED PLANT**

BY ANTON LANG, JAMES A. SANDOVAL, AND AHMED BEDRI

UNIVERSITY OF CALIFORNIA, LOS ANGELES

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Treatment with gibberellin induces bolting (stem elongation) and flower formation in many biennial and long-day rosette plants grown on a non-inductive temperature or photoperiod regime.[1] The gibberellins are products of certain strains of the fungus *Fusarium moniliforme* (sexual stage, *Gibberella fujikuroi*) and are not known to occur in any other plant. The physiological significance of their effect on bolting and flower formation may therefore be subject to some question. However, Phinney *et al.*[2] have recently shown that immature seeds or fruits of numerous angiosperms contain materials causing normal growth in certain dwarf mutants of maize (*Zea mays*) which otherwise respond only to gibberellin. If these gibberellin-like substances of seed plants regulate bolting and flowering in a manner similar to the *Fusarium* gibberellins, it becomes highly probable that they function as normal. native regulators of these processes in the plant. The present paper provides evidence that bolting and flowering in rosette plants can indeed be induced by the application of gibberellin-like materials from a seed plant.

Material and Methods.—Endosperm of *Echinocystis macrocarpa* Greene, a mem-

ber of the Cucurbitaceae, was used as source for gibberellin-like substances from seed plants. Phinney and co-workers[3] found this material to be particularly rich in such substances. The activity appears at very early stages in seed development, seeds 2–8 mm. in length containing active endosperm, and increases to a high level until the endosperm has been absorbed by the embryo.[4] At least two substances seem to share in the total gibberellin-like activity of the endosperm.[5]

The endosperm, which has the consistency of soft jelly, was collected from the seeds by suction. Part of it was used in the original form or was diluted with water. Another part was purified by procedures developed by West and Phinney.[5] The original endosperm was diluted with an equal part of water, the active material adsorbed to charcoal and eluted with 95 per cent acetone (volume equal to that of original endosperm), and the acetone was evaporated under stirring and fanning, the greater part at room temperature, the last traces under heating to approximately 50° C. This procedure results in a twenty fold concentration of activity. Still another part of the original endosperm was concentrated tenfold by direct evaporation. The resulting syrupy material proved to be rather toxic to the test plants, but a highly active and little toxic liquid fraction could be separated by centrifugation.

The test plants used were a biennial strain of *Hyoscyamus niger* L. and the long-day plant *Samolus parviflorus* Raf. Both plants bolt and flower promptly in response to gibberellin treatment (Lang, 1957).[1] *Hyoscyamus* was grown at temperatures of 18° C. or above and in long days (18 hours of light daily or more), *Samolus* in 9-hour short days. Under these conditions control individuals grew in the habit of rosettes and remained strictly vegetative.

The endosperm preparations were applied to the center of the rosette and later, after elongation had begun, to the growing region of the stem. For comparison, plants were treated with aqueous solutions of gibberellin A_3 (gibberellic acid). The amount of preparation or solution applied at a time was 0.2 ml., and applications were made either once daily or three times a week. Exceptions will be noted below (see Table 2). The endosperm preparations and gibberellin A_3 solutions contained a wetting agent (Polyglycol-31, Dow) at a rate of about 0.5–1 ml. per liter.

FIG. 1.—Biennial *Hyoscyamus niger*, non-cold-treated. *Left*, plant treated with 2 μg. of gibberellin A_3; *right*, treated with *Echinocystis* endosperm ("original"). Treatment daily; greenhouse conditions; photograph taken 48 days after start of treatment.

Experimental.—The results of an experiment with *Hyoscyamus* are summarized in Table 1; plants treated with *Echinocystis* endosperm and with gibberellin A_3 are shown in Figure 1. All preparations of the endosperm, when applied to the plants, caused bolting and flower formation. From the data in Table 1 it appears that 0.1 ml. of the original endosperm has a bolting- and flower-inducing action similar to that of 20 μg. of gibberellin A_3. The same flowering response was

caused by 0.2 ml. of the endosperm as by 20 μg. of gibberellin; dilution to one-half did not reduce the effect, while dilution to one-fifth reduced it to the level of 5–10 μg. of gibberellin. Tenfold concentration, on the other hand, did not result in an appreciable increase in the response, and 20 μg. of gibberellin A_3 were—under the conditions of this experiment—the optimum amount for the flowering response of *Hyoscyamus*. The height of the endosperm-treated plants, in contrast, lagged behind those treated with gibberellin. Plants that received 20 μg. of gibberellin per application reached an average height of 121 cm.; those treated with the original endosperm or a tenfold concentrate attained only about 1 meter.

TABLE 1

EFFECT OF *Echinocystis* ENDOSPERM AND OF GIBBERELLIN A_3 ON BOLTING AND FLOWERING IN NON-COLD-TREATED BIENNIAL *Hyoscyamus**

Treatment		No. Plants Flowering	No. Plants Vegetative	Days to Bolting	Height (Cm.)
Endosperm	10×†	9	0	10	103
	original, undiluted	9	1	11	99
	original, 1/2×	9	1	11	99
	original, 1/5×	5	5	10	88
Gibberellin A_3	20 μg./applic.	9	1	9	121
	10 μg./applic.	5	3	10	107
	5 μg./applic.	3	5	12	91
	2 μg./applic.	3	4	14	87
Control		0	10	..	0

* Application 3× a week; treatment under constant temperature conditions (22°–23° C.), 18 hours of light (fluorescent tubes) daily.

† Concentrated twenty fold (charcoal, acetone; see "Materials and Methods"); diluted with equal volume of water.

According to the work of Ritzel,[4] the endosperm of one *Echinocystis* seed induces in *dwarf-1* maize a growth response similar to that induced by 3.6 μg. of gibberellin. Since one *Echinocystis* seed contains, on the average, 0.6 ml. of endosperm, this effect corresponds to a content of 0.6 μg.-equivalent of gibberellin per 0.1 ml. of endosperm. Thus, in comparison to gibberellin, the efficiency of the *Echinocystis* endosperm in inducing bolting and flower formation in *Hyoscyamus* seems to be relatively greater than its growth-promoting capacity. It must be stressed, however, that the above estimates are only rough approximations. A wider range of endosperm and gibberellin concentrations, a larger plant material, and a wider variety of experi-

TABLE 2

EFFECT OF *Echinocystis* ENDOSPERM ON BOLTING AND FLOWERING IN *Samolus parviflorus* GROWN ON SHORT DAY

Treatment	No. Plants Flowering	No. Plants Vegetative	Days to Bolting	Days to Visible Buds
Endosperm, original, applied 1× daily	5	0	6	22
Endosperm, original, applied 3× daily	5	0	5	20*
Endosperm, 10× conc. (by evaporation)	2†	3	‡	37†
Endosperm, supernatant of tenfold conc., 0.1 ml. applied 2× daily	5	0	6	25
Endosperm, 20× conc. (charcoal, acetone), applied 16 days	5†	0	5	36†
Endosperm, 20× conc., applied 8 days	5	0	6	19
Gibberellin A_3, 2 μg. daily	5	0	11	20
Control	0	10	..	...

* Plants with normally developed main axis only.

† Flowering from lateral buds only; main axis killed or injured.

‡ Growing region of rosette killed before elongation.

mental conditions must be examined in order to establish more accurately the effectiveness of the *Echinocystis* endosperm in the various gibberellin-controlled plant responses. The main point, at the present, is that the active material from *Echinocystis* endosperm produces quite a similar bolting and flowering response in biennial *Hyoscyamus* as do the known gibberellins.

The results with *Samolus* (Table 2, Fig. 2) were similar to those with *Hyoscyamus*. In *Samolus*, only the original (undiluted) endosperm and concentrated prepa-

FIG. 2.—*Samolus parviflorus*, short-day-grown. *Left*, plants treated three times daily with *Echinocystis* endosperm ("original"); *right*, plants treated daily with 2 μg. of gibberellin A_3.

rations were tested. All induced bolting and flowering, but the effect of the concentrated preparations was not superior to that of the original endosperm, suggesting that the latter already contained an amount of active material(s) sufficient for the optimal response. Some of the preparations had toxic properties and killed or injured the tips of the treated plants. However, the majority of the injured plants flowered nevertheless, producing inflorescences from axillary buds in the leaf rosette.

Conclusions.—Our results show that the endosperm of *Echinocystis* is capable of inducing rapid bolting and flowering in a biennial plant which had been grown without exposure to low temperatures, and in a long-day plant grown under short-day conditions. This is the first time that a native material from a higher plant has been found to induce flowering in cold-requiring and photoperiodic plants under strict non-flowering conditions in a consistent and reproducible manner. Previous instances of chemical induction or promotion of flower formation with natural or synthetic chemical substances were limited to special cases or to plants which were maintained at threshold conditions for flowering (see review in Lang, 1957).[1]

The endosperm of *Echinocystis* contains materials which in another test—the growth response of certain maize dwarfs—behave precisely like gibberellins.[2] In procedures for partial purification and concentration, these materials and the flower-

inducing materials of the endosperm exhibit identical behavior. On the other hand, gibberellins are the only substances which cause normal growth in the maize dwarfs and bolting and flowering in cold-requiring and long-day plants. In view of these facts, it seems safe to conclude that the substances in the *Echinocystis* endosperm which promote growth in the maize dwarfs and which induce bolting and flowering in *Hyoscyamus* and *Samolus* are identical. This means that the gibberellin-like materials which exist in seed plants are capable of regulating bolting and flower formation, and it may be assumed that they play a crucial part in the normal regulation of these processes in the plant and may be involved in their control by low temperature and day length. The details of the function of these materials in bolting and flower formation as well as their chemical nature must await further work on the part of plant physiologists and chemists.

Summary.—Application of endosperm of *E. macrocarpa* (Cucurbitaceae) caused prompt bolting and flowering in biennial *H. niger* and in the long-day plant *S. parviflorus* grown under non-inductive conditions. The effect can be attributed to the gibberellin-like materials which are present in this endosperm. It is concluded that gibberellin-like materials participate in the processes of flower formation in plants and their control by low temperature and photoperiod.

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[1] A. Lang, *Naturwissenschaften*, **43**, 257, 284, and 544, 1956; these PROCEEDINGS, **43**, 709, 1957; F. Lona, *Nuovo giorn. botan. ital.*, N.S., **63**, 61, 1956; R. Bünsow and R. Harder, *Naturwissenschaften*, **43**, 479 and 527, 1956; S. H. Wittwer and M. J. Bukovac, *Quart. Bull. Michigan Agr. Exptl. Sta.*, **39**, 469, 1957; *Science*, **126**, 16, 1957.

[2] B. O. Phinney, C. A. West, M. Ritzel, and P. Neely, these PROCEEDINGS, **43**, 398, 1957.

[3] Personal communication.

[4] M. Ritzel, *Plant Physiol.*, **32** (Suppl.), xxxi, 1957, and personal communication.

[5] C. A. West and B. O. Phinney, *Plant Physiol.*, **32** (Suppl.), xxxii, 1957, and personal communication.

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*DISPROPORTIONATE SYNTHESIS OF DNA IN A POLYTENE CHROMOSOME REGION**

BY G. T. RUDKIN AND S. L. CORLETTE

INSTITUTE FOR CANCER RESEARCH AND LANKENAU HOSPITAL RESEARCH INSTITUTE, PHILADELPHIA, PENNSYLVANIA

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Recent studies of the morphology of the giant polytene chromosomes in dipteran larvae[1] have emphasized the variability from tissue to tissue and from one developmental stage to another of localized swellings: Balbiani rings, bulbs, and puffs. The correlation between the secretory activity in certain cells and the appearance and disappearance of puffs at specific loci in those cells had led many authors[1, 3] to the hypothesis that the genes at the locus of the puff may be actively controlling

the secretory process. In most cases it is sufficient to assume, on the basis of available evidence, that the swelling brings about a dilution of deoxyribonucleoprotein, the activity of which is increased by the consequent freer flow of metabolites and greater exposed surface area in the already thousand fold polytene chromosome. We have found that there is no significant increase in nucleoprotein when one of the regions in a *Drosophila* salivary gland chromosome puffs.[2] Breuer and Pavan,[3] on the other hand, observed in stained preparations what appeared to be an accumulation of strongly basophilic, Feulgen-positive material at the site of a regressed puff in prepupae of *Rhynchosciara angelae*. With the aim of finding out whether or not a disproportionate increase in DNA had taken place during the growth of the chromosomes, we have measured the nucleic acid and protein content of puffed and unpuffed chromosomes in material sent to us in the living state from Brazil by Professor Pavan.[4] We used the total absorbancy at 257 mμ in the ultraviolet, determined by a photographic procedure,[5] as a measure of the quantity of absorbing material in a chromosome, and we estimated the chemical constituents by extracting the chromosomes with nucleases and with hot trichloracetic acid. Our first results confirm the conclusions of the earlier authors so strikingly that they are presented here as a preliminary note.

Experimental.—Rhynchosciara angelae occurs in families of several hundred larvae developing in almost perfect synchrony. Individuals sacrificed on successive days form a developmental series typical of the whole family. As Breuer and Pavan showed, a puff (puff *a*) is formed near the tip of the B chromosome in the nuclei of the proximal salivary gland at the time of onset of the spinning of the puparium. As the spinning progresses, the puff swells to maximum distention, then shrinks and leaves in its place a densely staining mass of chromatin. During the recession of the *a* puff, the chromatin just distal to it becomes densely staining, then forms a small puff (puff *b*), which also recedes, to become an even more densely staining mass. We have completed measurements on three chromosomes from the proximal salivary glands from each of three larvae, one before, one during, and one after the maximum development of puff *a*.

Salivary glands dissected out on ice in the larva's own haemolymph were fixed 4–24 hours in 45 per cent aqueous acetic acid at 0° to −5° C. The proximal parts were squashed onto albuminized quartz slides under siliconed glass cover slips. The slides were frozen on dry ice, the covers snapped off, and the slides subjected to freezing substitution with absolute alcohol at −40° C. The preparations were mounted on a solution of 45 per cent zinc chloride in glycerine[6] under quartz cover slips for ultraviolet microspectrophotometry.

For treating the chromosomes in aqueous media, the mounting fluid was removed with 95 per cent alcohol, the specimens were rehydrated and treated with ribonuclease (RNAase), then dehydrated through alcohols and remounted in zinc chloride–glycerine for further microspectrophotometry. This procedure was repeated for treatment with deoxyribonuclease (DNAase).[5] The final treatment was extraction of all remaining nucleic acids with 5 per cent trichloracetic acid for 15 minutes at 90°C. (hot TCA), followed, as before, by microspectrophotometric measurement.

Preliminary tests of the methods showed that the reagents were active. A second application of enzyme solution did not remove additional absorbing sub-

stances from the chromosomes, nor did incubation in distilled water under the same conditions. Ribonuclease completely removed ribonucleic acid (RNA) from the cytoplasm on all preparations. Deoxyribonuclease did not remove significant amounts of absorbing material from the cytoplasm when it was applied before RNAase; it did remove over 90 per cent of the absorbancy from an RNAase-treated small chromosome that probably came from one of the cells near the salivary gland duct. Chromosomal material extracted with both enzymes no longer stained with azure B but was found to have an absorption peak near 260 mμ, showing that nucleic acid derivatives were present. After extraction with hot TCA, the absorption maximum was close to 280 mμ.[9] It should be emphasized that the data derived from these treatments are not used here to calculate the percentage composition of the chromosomes but to compare relative amounts extracted from them.

We divided the distal portion of chromosome B into six adjacent segments. The most distal segment, I, did not puff and extended from the tip of the chromosome to a prominent band marking the limit of puff *a* (see above). The next two segments, II and III, were the sites of the two puffs, *a* and *b*, respectively. The next three segments, IV, V, and VI, did not puff. The absorbancy in section V was so high at most stages that it could not be measured under the conditions of this experiment. It was extremely difficult to obtain preparations of puffed chromosomes which were sufficiently stretched to permit accurate demarcation of all regions and at the same time not squashed so much as to blur the boundaries near the puff itself. Thus segments I–III were necessarily lumped together in the puffed chromosome; for comparison with it, they have been lumped in the other two chromosomes in one row of Table 1.

TABLE 1

INTEGRATED ABSORBANCY AT 257 mμ OF SEGMENTS OF THREE SALIVARY GLAND B CHROMOSOMES OF *Rhynchosciara angelae* AT DIFFERENT STAGES IN DEVELOPMENT

		Before Treatment				After Treatment with RNAase, DNAase, and Hot TCA			
Stage on Day*		Pre-puff 9	Puff 13	Post-puff 15	Ratio Post/Pre	Pre-puff 9	Puff 13	Post-puff 15	Ratio Post/Pre
Segment	I	26.5	...	51.0	1.9	11.1	...	11.8	1.1
	II	**19.4**	†	**76.3**	**3.9**	**6.9**	†	**13.4**	**1.9**
	III	**18.1**	†	**44.9**	**2.5**	**7.0**	†	**6.6**	**0.9**
	I–III	**64.0**	**111.1**	**172.2**	**2.7**	**25.0**	**29.3**	**31.8**	**1.3**
	IV	17.0	25.1	35.0	2.1	8.0	4.1	6.6	0.8
	VI	52.5	77.1	106.3	2.0	23.7	18.3	24.8	1.0

* Days after arrival of larvae in Philadelphia, U.S.A., from São Paulo, Brazil. The spinning of the puparium began on day 11, pupation began on day 16.

† Maximum development of puff in segment II, small puff present in segment III. No puffs occur in other segments during the period covered. Figures in boldface type refer to regions in which puffs develop.

Results.—Extra synthesis of ultraviolet-absorbing material in the puff regions is shown by comparison of the two extreme stages (Table 1, fifth col.). The integrated absorbancy has increased twice as much in the region of the first puff (about fourfold) as in the regions which did not puff (about twofold). Intermediate values are found in the stage when the puff is present, although lumping of non-puff with puff regions obscures the magnitude of the individual values. In the region of puff *b* the over-all ratio of 2.5 fold is intermediate between that for puff *a* and for non-puff regions.

The residue after hot TCA extraction contains only the protein not extracted by the cold acetic acid fixative. It is possible to compare the contribution to total

absorbancy by this fraction in the chromosomes. Oddly enough, the values for the non-puffing regions are approximately the same in all the chromosomes (Table 1). Again, a twofold increase is found in the puff *a* region, but the significance of this difference is questionable because of the very low absorbancies encountered in the chromosome residue. Thus nucleic acids must account for nearly all the differences in total absorbancy found in untreated, fixed chromosomes.

The distinction between ribose and deoxyribose types of nucleic acids is still a very difficult cytochemical problem.[10] We have attempted it here by first treating with RNAase, then with DNAase before hot TCA. If the enzymes were completely effective, the difference between the absorbancy after RNAase and that after hot TCA should be a measure of DNA, and there should be little or no material left after DNAase to be extracted by the TCA.

In fact, the RNAase treatment did not significantly change the total absorbency of these chromosomes. This could mean that there is not enough RNA to be detected by our methods; that some, or all, of it was removed in the fixation procedure; or that it is bound in such a way that it is not subject to attack by the enzyme. In either of the first two cases the difference between RNAase and TCA treatments can be attributed to DNA. This difference was obtained for each chromosome segment with the same result as before: the region of puff *a* synthesized about twice as much DNA as did regions without puffs, and the region of the small puff *b* was intermediate between the two (Table 2).

DNAase had a small but measurable effect on total absorbencies; it removed between 15 and 30 per cent of the absorbency of the RNAase residue that came out in hot TCA. This treatment divides the DNA of the previous paragraph into two portions, only one of which is removed by the enzyme. Of the two portions, the one removed by DNAase and thus most likely to represent only DNA is estimated by the difference between the total absorbency values after RNAase and after DNAase treatments. This difference in some of the pre-puff stage chromosome segments is so close to the errors in the original measurements themselves that it is often equal to, or smaller than, its own error. The other portion, not removed

TABLE 2

DIFFERENCES BETWEEN INTEGRATED ABSORBANCIES AT 257 mμ OF SAME CHROMOSOMES AS IN TABLE 1 TREATED AS INDICATED IN COLUMN HEADINGS

		Treatment							
		RNAase minus Hot TCA				DNAase minus Hot TCA			
Stage on Day*		Pre-puff 9	Puff 13	Post-puff 15	Ratio Post/Pre	Pre-puff 9	Puff 13	Post-puff 15	Ratio Post/Pre
Segment	I	17.7		41.7	2.4	12.0		25.6	2.1
	II	12.4	†	62.8	5.1	11.3	†	37.9	3.4
	III	11.7	†	35.1	3.0	9.0	†	23.3	2.6
	I–III	41.8	85.4	139.6	3.4	32.3	50.6	87.0	2.7
	IV	10.3	22.8	25.2	2.4	7.5	14.2	16.1	2.2
	VI	24.6	44.1	63.9	2.6	21.8	38.6	41.2	1.9

		Treatment			
		RNAase Minus DNAase			
Segment	I	5.7		16.1	2.8
	II	1.1	†	24.9	22.6
	III	2.7	†	11.8	4.3
	I–III	9.5	34.8	52.8	5.6
	IV	2.8	8.6	9.1	3.3
	VI	2.8	5.5	32.7	11.7

* See Table 1 for notes.

by DNAase action, is measured by larger and more reliable differences. The pattern of the results for both portions (Table 2) is consistent with the foregoing data. Whichever criterion is used to identify DNA, we must conclude that it increased in these puff regions more than it did in non-puffing regions during the formation and regression of the puffs.

Discussion.—The cytological picture presented by Breuer and Pavan, though striking, cannot alone suffice to prove a disproportionate increase in DNA. The cytological measurements reported here supply the necessary support of such an interpretation. They show that the DNA in the puff region at least doubles with respect to non-puffing regions.

The rule that the DNA content of a nucleus is directly proportional to a whole number of chromosome sets is derived from measurements made on whole nuclei.[11] Since the region involved in the present case (puff *a*) is shorter than one one-hundredth of the total length of all the chromosomes, the twofold increase we observed there would add less than 1 per cent to the total nuclear DNA. A difference of this magnitude could not be detected by existing methods. Clearly, the disproportionate DNA synthesis on these chromosomes could not be deduced from measurements of total DNA per nucleus. This fact raises serious difficulties for inferences as to the relation of DNA metabolism to gene action derived from the so-called DNA constancy rule.

On the other hand, the present case is not inconsistent with the theory that there is a DNA component replicated in a regular fashion in these chromosomes. This "constant" component is then especially active in puffed regions, where it is augmented by the "extra" DNA in the case studied here. The relation between this extra amount and the material originally present is a question now open to experimental attack.

* Aided by Grant C-1613 to Dr. Jack Schultz from the National Cancer Institute of the National Institutes of Health, Public Health Service, and by an institutional grant from the American Cancer Society.

[1] See W. Beerman, *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 217, 1956, for review.

[2] G. T. Rudkin, *Genetics*, **40**, 593, 1955.

[3] M. E. Breuer and C. Pavan, *Chromosoma*, **7**, 371, 1955.

[4] We wish to express our gratitude to Professor Pavan and M. E. Breuer for their generous co-operation.

[5] G. T. Rudkin, J. F. Aronson, D. A. Hungerford, and J. Schultz, *Exptl. Cell Research*, **9**, 193, 1955.

[6] The $ZnCl_2$ increases the refractive index of the glycerine and thereby reduces non-specific light loss for microspectrophotometric measurements (see G. T. Rudkin and S. L. Corlette, *J. Biochem. Biophys. Cytol.*, **3**, 821, 1957).

[7] Worthington's crystalline, salt-free ribonuclease, 0.05 per cent in glass-distilled water brought to pH 6.6 with NaOH. Treatment for 2 hours at 37° C.

[8] Worthington's crystalline desoxyribonuclease 1× crystallized, 0.1 mg/cc in 0.003 *M* $MgSO_4$ adjusted to pH 6 with NaOH. Treatment for 2 hours at 37° C.

[9] The absorption spectra were measured with a recording photoelectric "ultramicrospectrograph" described by T. Caspersson, F. Jacobsson, G. Lomakka, G. Svenson, and R. Säfström, *Exptl. Cell Research*, **5**, 560, 1953.

[10] See B. P. Kaufmann, M. R. McDonald, and H. Gay, *J. Cellular Comp. Physiol.*, **38** (Suppl 1), 71, 1951, and note 11.

[11] H. Swift, *Intern. Rev. Cytol.*, **2**, 1, 1953.

*HYPOTHESIS FOR THE MECHANISM OF ACTION OF CHYMOTRYPSIN**

By F. H. Westheimer

MALLINCKRODT CHEMICAL LABORATORIES OF HARVARD UNIVERSITY

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An interesting and provocative hypothesis for the detailed mechanism of action of chymotrypsin can be developed on the basis of the assumption that the protein near the active site in the enzyme is arranged in an alpha helix.[1] Although this assumption is unproved, the mechanism derived from it fits most of the known data and is specific in its predictions. In particular, it is consistent with the fundamental data on the enzyme given on the following page.

Serine is essential to the functioning of chymotrypsin, and the amino acid sequence[2] in the neighborhood of the active site is presumably

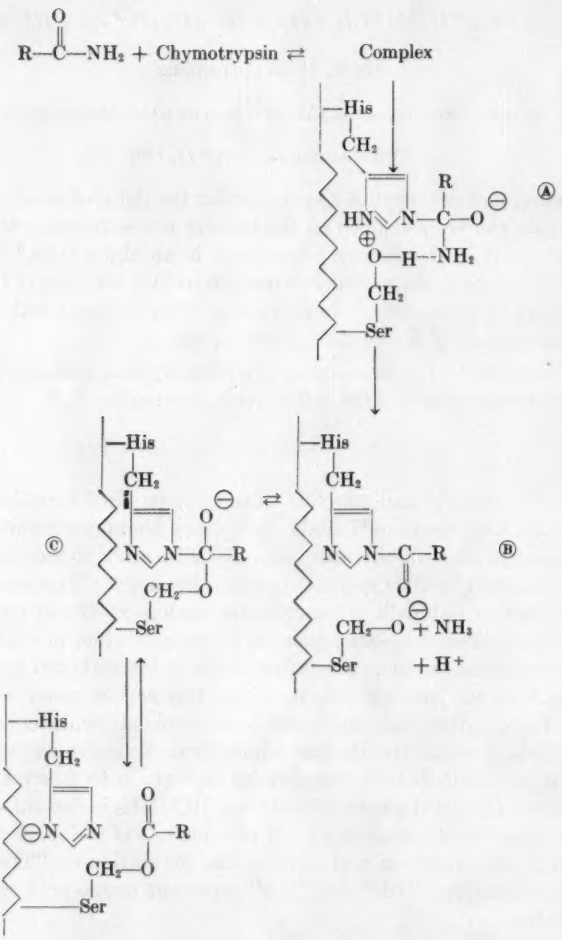
Gly—Asp—Ser—Gly—Glu—Ala—Val.

Further, kinetic evidence[3] indicates that histidine is required for enzymatic activity, and Schaffer and his co-workers[2] found indications of histidine somewhere near serine in a degradation product of chymotrypsin. The function of the imidazole residue of histidine is implied by the experiments which Bender and Turnquest[4] and Bruice and Schmir[5] carried out with non-enzymatic analogs of the enzymatic systems; presumably the unshared electron pair on a nitrogen atom of imidazole (see accompanying equations) attacks the carbon atom of the carbonyl group of an ester or amide, to form an intermediate, A, where this carbon atom is tetracovalent. The intermediate, A, then loses an alcohol or ammonia molecule to yield an acylated imidazole, B, which constitutes the first relatively stable intermediate in the process. The acylated imidazole in turn transfers its acyl group to a serine residue of the enzyme[6] to form C. In the equations shown, $RCONH_2$ is the substrate (e.g., the amide of N-benzoyl L-phenylalanine). A possible role of serine, as a proton donor, is illustrated by the equations and explained in the section on "The Intermediate, A." In these equations, "His" and "Ser" represent amino acid residues without their side-chains.

This sequence of reactions can be explained in terms of a postulated alpha-helical structure for chymotrypsin, together with the assumption that histidine is in the fifth position with respect to serine. The essential points of the mechanism are illustrated by a model for a right-handed helix, constructed from the following peptide (where the free carboxyl group is that of valine):

—*X*—	*X*—	*X*—	His—	*X*—	Gly—	Asp—	Ser—	Gly—	Glu—	Ala—	Val—
12	11	10	9	8	7	6	5	4	3	2	1

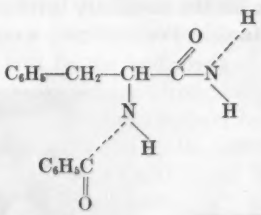
The amino acid molecules indicated here by *X* are at present unknown; they have been represented in the model by glycine. The right-handed helix automatically places the serine, histidine, and aspartic acid residues in juxtaposition; the essential reactions, the pH-rate profile, the stereospecificity, the structural specificity of the enzyme, and several features of inhibition can now be explained.



The Michaelis Complex.—The Michaelis constants for various substrates[7, 8] and the inhibitor constants[8] for chymotrypsin are independent of stereochemical or structural features, so long as the compound in question contains an aromatic residue. Thus the Michaelis constant for the methyl ester of phenyl propionic acid is as small (i.e., as favorable) as that for a specific substrate such as N-benzoyl L-phenylalanine amide, and the inhibitor constants for benzamide and for indole are not much larger.[9, 10] Further, the inhibitor constant for *d*-phenyl lactic acid is about the same as that for the *l*-isomer,[7] and the inhibitor constants for the methyl esters of *d*- and of *l*-tyrosine are approximately equal. The enzyme model (which is highly specific with respect to the chemical intermediate A) is less specific with respect to the Michaelis complex, but a reasonable formulation would include placing the phenyl group between the methyl group of the alanine residue (No. 2) and the isopropyl group of valine (No. 1) in the amino acid chain, and hydrogen-bond-

ing the carbonyl group (if there is one in the inhibitor) with the hydroxyl group of serine. Such a complex is shown (for N-benzoyl L-phenylalanine) in Figure 1. Presumably, the binding of the phenyl group also includes contributions from amino acids in the chymotrypsin chain which are not now specified, or from other chains; the fact that there is a difference in Michaelis constants for α and δ chymotrypsins[8] suggests that an additional chain is involved, since these two modifications of the enzyme differ in a region far from the active site.[11] A complex of the type shown conforms with experiment, in that it does not distinguish between stereoisomers or between an ester and a ketone[10] or between an ester of phenyl propionic acid and one of N-benzoyl phenylalanine. However, it should be noted that, in this complex, the carbon atom of the carbonyl group is only about an angstrom from one of the nitrogen atoms of the imidazole ring.

The Intermediate, A.—The intermediate, A, is shown in Figure 2 and an "exploded" model of A is shown in Figure 3. In Figures 2 and 3, the carbonyl carbon atom of the amide group has been bonded chemically to a nitrogen atom of the imidazole residue. The serine hydroxyl group is hydrogen-bonded to the nitrogen atom of the amide group. The phenyl group is still lodged between the methyl and isopropyl groups of the alanine and valine residues. The close and accurate "fit" near the reaction site can be judged in part from Figure 2; however, the essential features of the mechanism are more easily seen in Figure 3, where the C_6H_5CO group of benzoyl phenylalanine amide and one of the hydrogen atoms of the amide have been removed to reveal the essential hydrogen bonding in A; in the formula



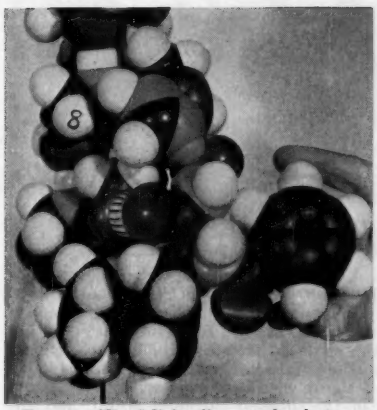


FIG. 1.—The Michaelis complex between chymotrypsin and the amide of N-benzoyl L-phenylalanine.

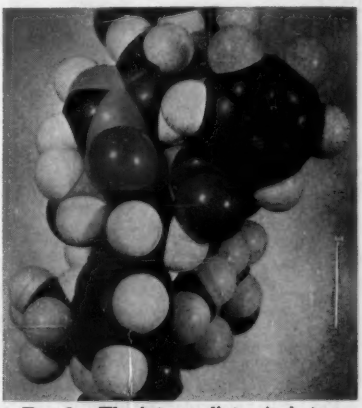


FIG. 2.—The intermediate, A, between chymotrypsin and the amide of N-benzoyl L-phenylalanine.

the long dotted bonds are those broken in the "exploded" model. The hydrogen atom of serine, which binds the amide nitrogen atom, has been striped for better identification. The transfer of this hydrogen atom to the amide nitrogen during hydrolysis of the amide allows for the formation of an alcoholate ion (hydrogen-bonded to ammonia) rather than an amide ion. The transfer of a proton from an alcohol to the nitrogen atom of an incipient amide ion is not inherently preferred to the transfer of a proton from a neighboring water molecule; the favorable geometry of the complex, however, may allow considerable catalysis by the serine residue. Although no precise analogy to the present example is available, the rapid hydrolysis[12] of esters of 2-hydroxytriptycene is somewhat similar, and the rapid hydrolysis[13] of phthalamic acid suggests the efficiency of accurate geometric placement in general acid catalysis. On the other side of the molecule, the hydrogen atom of the benzoyl amide group has also been striped for better identification; this hydrogen is bonded to the carboxylate anion group of the aspartic acid in position 6 of the peptide chain. The molecules fit so tightly together that no hole larger than about a cubic angstrom is formed between the substrate and the helix.

The complex shown in Figure 3 accounts for most of the essential features of the reaction and is therefore discussed in some detail. Since esters are hydrolyzed by chymotrypsin much more rapidly than the corresponding amides, the rate-controlling step in the hydrolysis of the latter must be the formation of the intermediate A; this conclusion follows because the acyl imidazole complex, B, formed from an ester is identical with that obtained from the corresponding amide. The model shown in Figure 3 accounts not only for the chemistry involved but also for special structural and stereochemical features. For example, a corresponding model from N-

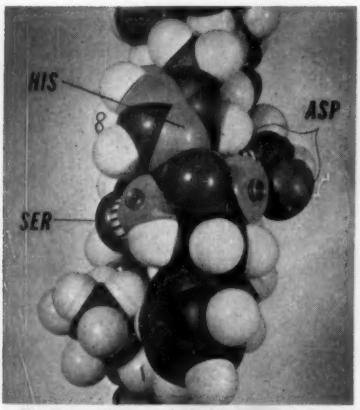


FIG. 3.—An exploded view of intermediate A. The two hydrogen atoms which participate in hydrogen bonding have been striped for better identification.

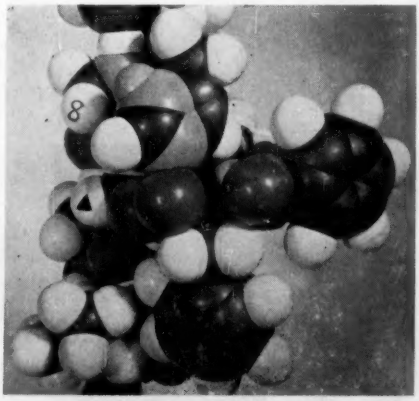


FIG. 4.—The intermediate, C, between chymotrypsin and N-benzoyl L-phenylalanine. One of the hydrogen atoms of the methylene group of serine has been marked for identification with a triangle. The carbon atom which was originally part of the amide carbonyl group is now hidden behind the oxygen atom of serine, the oxygen atom of the original carbonyl group, and the imidazole nitrogen atom. *White* = hydrogen; *light gray* = nitrogen; *dark gray* = oxygen; *black* = carbon.

benzoyl D-phenylalanine amide is nowhere near so satisfactory as that here shown; either the hydrogen bond to the aspartate anion must be sacrificed, or the fit of the phenyl group, or a large hole must be formed between the peptide chain and the substrate. Further, a substrate (e.g., the methyl ester of phenyl propionic acid) which does not have a substituent (like the N-benzoyl amino group) alpha to the carboxamide group cannot be bound at the aspartate ion at all and therefore will not react so fast as a proper substrate. Incidentally, the close "fit" occurs only with the right-handed coil shown in the figures and cannot be duplicated with a left-handed helix.

Although the simplest postulate places the histidine, serine, and aspartic acid residues in a close relationship, topologically as well as sterically, alternate possibilities may be advanced. A similar mechanism (with the same roles for serine and aspartic acid) can be formulated if the histidine is in an adjacent coil of the protein, held in place (for example) by disulfide bonds. Nevertheless, the principle of parsimony suggests that the present mechanism be critically examined before more complex ones are considered.

The Acylated Serine.—When intermediate A decomposes by ejecting a molecule of ammonia, the acyl imidazole intermediate, B, is formed. However, it is formed in the presence of a nearby alcoholate ion, and the stereochemical arrangement is perfect for further reaction. The transfer of an acyl group from nitrogen to oxygen is a well-known one and occurs readily in acylated serine;[14] both the stereochemical and the chemical arrangements here are ideal for rapid reaction. The intermediate, C, which must be formed during the transformation of the acyl imidazole to the acyl serine, is shown in Figure 4. The reaction is reversible;[14] and the final step in the enzymatic sequence will be the hydrolysis of an acylated imidazole intermediate (i.e., B) by water or hydroxide ions from solution.

The pH Effects.—The activity of chymotrypsin rises to a maximum in alkaline solution,[3] as if the enzyme had a pK of 6.7; the activity is maintained in solutions of pH 9. The mechanism is in accord with these facts. The free unprotonated imidazole is needed to form the intermediate, A. The carboxyl group of the aspartic acid residue is necessarily ionized in the pH region in question; the pK of a carboxyl group is never displaced[15] in a protein by a sufficient amount to allow an appreciable concentration of the un-ionized carboxyl group at a pH of, say, 8.5. The equilibrium between acylated serine and acylated imidazole is probably pH-dependent, with the acyl group displaced to the serine at low pH. Either this effect or a requirement for hydroxide ion in the last step of the reaction can account for the relative stability[6] of acylated chymotrypsin at pH 5 as compared with that at pH 7 or 8.

A much more subtle pH effect, in this instance on the efficiency of inhibition, can also be partially explained. Foster and Niemann[16] found that ionized inhibitors, such as β-phenyl propionate ion, are less efficient than un-ionized ones, such as β-phenyl propionamide. They therefore postulated that the enzyme has a negatively charged group in the neighborhood of the active site. This group is here identified as the carboxylate ion group of aspartic acid. But they also noted that an anionic inhibitor becomes more tightly bound when the pH is lowered and that this change occurred between pH 6.9 and 7.9. This pH is too high to involve the ionization of the inhibitor itself (i.e., phenyl propionate ion), and therefore they assumed that

the pK of an anionic site on the enzyme falls in this region. This conclusion seems improbable. But the pK of the imidazole group is in this region, and in the inhibitor complex the electrostatic effect of a positively charged imidazole group would largely cancel out the electrostatic effect of the negatively charged aspartate ion and lead to the results that Niemann and Foster observed. The explanation for the pH effects on the inhibition by tryptamine, however, is still lacking.

Structural Effects.—The model easily accounts for the fact that the esters of phenylalanine and tyrosine are poor substrates. The amino acid residues,

$$C_6H_5CH_2—\underset{\displaystyle \overset{|}{\underset{\displaystyle \overset{+}{NH_3}}{|}}}{CH}—CONH_2$$

carry a positive charge in the appropriate pH range; in the intermediate, A, this charge will be in close proximity to the positive charge on the imidazole residue. Other structural effects have already been discussed.

Limitations.—The model accounts for most of the chemistry of chymotrypsin quite well. The assumption of the alpha helix and the prediction of the position of histidine are unproved; if these predictions are verified, the model accounts for the reactions, for the gross chemical specificity, and for the stereochemical specificity. It does not, without refinements, account for all the known effects of variations in the structure of the substrate. For example, the model will accommodate the methyl ester of L-phenyl lactic acid better than the D-isomer, in accordance with fact.[7] The L-isomer is a good substrate; but the theory does not account for the fact that the esters of N-benzoyl phenylalanine are better[7] by a factor of about 30. The hydrogen bond between the hydroxyl group of phenyl lactate and the carboxylate anion of the aspartate residue should be comparable to that between the benzamide group and the carboxylate anion.[17] If the model proves generally useful, perhaps some refinement will explain this fact (e.g., a water molecule "fits" well between the carbonyl oxygen of the benzamide group and the adjacent negatively charged oxygen atom). Another difficulty pertains to the rates of hydrolysis of substituted amides. The rates of hydrolysis of N-acetyl L-tyrosyl amide and of N-acetyl L-tyrosyl glycinamide are comparable,[8] but the rate of hydrolysis of acetyl tryptophane N-methylamide is slow,[18] and presumably much slower than that of the unsubstituted amide. This and similar problems remain for analysis, even if the general mechanism here presented is confirmed.

* The author is deeply indebted to Professor D. F. DeTar for helpful discussions of this mechanism. He should also like to thank the United States Rubber Company for the funds used to purchase the models.

[1] L. Pauling, R. B. Corey, and H. R. Branson, these PROCEEDINGS, **37**, 205, 1951.

[2] N. K. Schaffer, L. Semet, S. Harshman, R. R. Engel, and R. W. Drisko, *J. Biol. Chem.*, **225** 197, 1957; N. K. Schaffer, R. E. Engle, L. Semet, R. W. Drisko, and S. Harshman, *Federation Proc.*, **15**, 347, 1956; cf. also F. Turba and G. Gundlach, *Biochem. Z.*, **327**, 186, 1955; R. A. Oosterbaan, H. Janz, and J. A. Cohen, *Biochim. et Biophys. Acta*, **20**, 402, 1956; D. E. Koshland, Jr., and M. J. Erwin, *J. Am. Chem. Soc.*, **79**, 2657, 1957.

[3] L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287, 1956; B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187, 1955.

[4] M. L. Bender and B. W. Turnquest, *J. Am. Chem. Soc.*, **79**, 1652, 1656, 1957.

[5] T. C. Bruice and G. L. Schmir, *Arch. Biochem. and Biophys.*, **63**, 484, 1956; *J. Am. Chem. Soc.*, **79**, 1663, 1957.

[6] A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245, 1956.
[7] J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351, 1949.
[8] H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69, 1950.
[9] R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3370, 1955; R. J. Foster, H. J. Shine, and C. Niemann, *J. Am. Chem. Soc.*, **77**, 2378, 1955.
[10] S. Kaufman and H. Neurath, *J. Biol. Chem.*, **181**, 623, 1949; *Arch. Biochem.* **21**, 437, 1949.
[11] B. Meedom, *Acta Chem. Scand.*, **10**, 881, 1956.
[12] P. D. Bartlett and F. D. Greene, *J. Am. Chem. Soc.*, **76**, 1088, 1954.
[13] M. L. Bender, *J. Am. Chem. Soc.*, **79**, 1258, 1957.
[14] M. Bergmann, E. Brand, and F. Dreyer, *Ber. Deut. chem. Ges.*, **54**, 936, 1921; M. Bergmann and A. Miekeley, *Z. physiol Chem.*, **140**, 128, 1924; E. van Tamelen, *J. Am. Chem. Soc.*, **73**, 5773, 1951; D. F. Elliott, *Biochem. J.*, **50**, 542, 1952.
[15] E. J. Cohn and J. Edsall, *Proteins, Amino Acids and Peptides*, (New York: Reinhold Publishing Co., 1943).
[16] R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3365, 1955.
[17] S. Mizushima, M. Tsuboi, T. Schimanouchi, and Y. Tsuda, *Spectrochim. Acta*, **7**, 100, 1956.
[18] H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **73**, 3223, 1951.

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*MODIFICATION OF RADIATION-INDUCED INJURY BY POSTTREATMENT WITH OXYGEN**

RICHARD S. CALDECOTT, ELIZABETH BINDLOSS JOHNSON, D. T. NORTH, AND C. F. KONZAK†

DEPARTMENT OF AGRONOMY AND PLANT GENETICS, UNIVERSITY OF MINNESOTA AND THE BROOKHAVEN NATIONAL LABORATORY

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INTRODUCTION

In the past decade it has been demonstrated in a wide range of biological systems that the presence of oxygen during treatment with X-radiation enhances the degree of detectable injury over that which is observed when organisms are maintained anoxic during treatment. The observations have been explained by some radiation chemists as supporting the view that free oxygen combines with the radio-decomposition products of water to produce highy reactive radicals, which may then oxidize or reduce sensitive sites within the organism. Because at normal temperatures and pressures the half-life of such radicals is measured in terms of milliseconds, it should be impossible to enhance radiation-initiated damage by subjecting organisms to hyperoxygenation following X-radiation. The present report describes conditions under which radiation-initiated injury to dry dormant barley seeds may be modified by postradiation hydration in the presence of oxygen.

MATERIALS AND METHODS

Dormant seeds of Himalaya barley were selected for uniformity of size and for freedom from disease and mechanical injury, to minimize variation in germination and seedling development. The water content of the selected seeds was stabilized prior to irradiation by storing them at room temperature in desiccators over dry phosphorus pentoxide or saturated sodium chlorate. Under these storage condi-

tions, the embryo water content of seeds stored over phosphorus pentoxide was approximately 4 per cent, while that of seeds stored over sodium chlorate was approximately 16 per cent. The terms "dry" and "wet," as frequently used in this report, refer to seeds having an embryo water content of 4 and 16 per cent, respectively.

For treatment with X-rays the seeds were turned embryo up in lucite trays. The trays were placed on a turntable that was centered with reference to the beam and rotated during treatment at approximately $3^1/_2$ revolutions per minute at a distance of 9.7 inches from the target. The X-rays were generated with a Muller RT 100 Constant Potential facility that was operated at 100 kv. and 7 ma. The radiation was filtered through the 1-mm. beryllium window of the tube and the air space between the tube and the surface of the seeds. Under these conditions the seed received approximately 3,000 r per minute as measured by a Victoreen Integron.

For treatment with thermal and fast neutrons, the thermal column facility in the Brookhaven Reactor was utilized. The procedural details are described elsewhere.[1,2]

Within 30 seconds following X-ray treatment and 60 seconds following neutron bombardment, 100-seed samples were hydrated by plunging them into 175 ml. of boiled distilled water that was continually flushed with either commercial oxygen or nitrogen. At the outset of hydration, the seeds hydrated in the presence of nitrogen were assured reasonably anaerobic conditions by flushing the flasks of water for at least 5 minutes prior to seed introduction.

After removal from the hydration flasks, lots of 50 seeds were placed embryo up on moist germination paper in 6-inch Petri dishes. They were then grown in a temperature- and light-controlled growth room operated at 70° ± 2° F. with 18 hours of continuous illumination per 24-hour period. At the end of 6 days, individual seedling height measurements were made, and the data record the average heights of each treatment in per cent of control.

EXPERIMENTAL RESULTS

Initial efforts to modify the radiosensitivity of barley seeds clearly demonstrated that under certain conditions the expression of radiation-induced seedling injury may be greater when the irradiated seeds are hydrated in the presence of oxygen as compared with the injury manifest in those hydrated in the presence of nitrogen. The difference in the extent of injury following these two hydration treatments is related to (1) the duration of the hydration period; (2) the water content of the embryo; and (3) the ion density. Furthermore, the extent of radiation-induced injury as expressed following anaerobic hydration is not stable but can be modified by an additional period of aerobic hydration. These points as related to specific experiments are presented below.

Duration of the Hydration Period.—Experiments in which X-rayed barley seeds with an embryo water content of 4 per cent were hydrated in boiled distilled water through which either oxygen or nitrogen was continually bubbled showed that the resulting seedling injury was appreciably greater in those seeds hydrated in the presence of oxygen than in those hydrated in the presence of nitrogen. Studies in which the hydration period ranged from 5 minutes to 24 hours were conducted to determine the length of the hydration period necessary to produce the maximum effect. There was no significant modification of seedling height of unirradiated

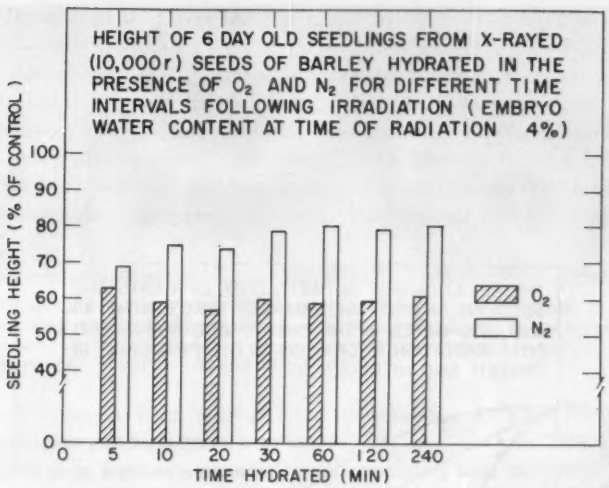
HEIGHT OF 6 DAY OLD SEEDLINGS FROM X-RAYED (10,000r) SEEDS OF BARLEY HYDRATED IN THE PRESENCE OF O_2 AND N_2 FOR DIFFERENT TIME INTERVALS FOLLOWING IRRADIATION (EMBRYO WATER CONTENT AT TIME OF RADIATION 4%)
SEEDLING HEIGHT (% OF CONTROL)
100
90
80
70
60
50
40
0
0 5 10 20 30 60 120 240
TIME HYDRATED (MIN)
O_2
N_2

FIG. 1.—Height of 6-day-old seedlings from X-rayed (10,000 r) seeds of barley hydrated in the presence of oxygen and nitrogen for different intervals following irradiation (embryo water content at time of radiation, 4 per cent).

controls that were hydrated in the presence of either gas for periods up to 12 hours. However, after 12 hours, oxygen toxicity caused injury to both control and irradiated seedlings.

In one experiment, dry seeds irradiated with 10,000 r of X-rays were hydrated for different periods of time ranging from 5 to 240 minutes (Figs. 1, 2). This study was repeated numerous times and clearly showed that maximum injury to seeds hydrated in the presence of oxygen takes place within a period of 30 minutes. For this reason, in most subsequent studies a single hydration period of 45 minutes was used. This period was arbitrarily chosen as one that would guarantee slightly more than the minimum period of hydration required and yet not be wasteful of time and effort.

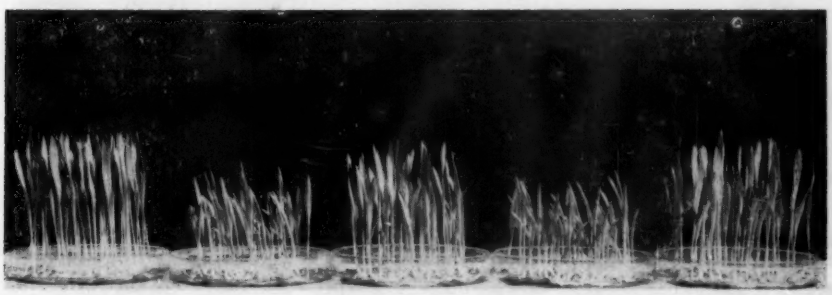

FIG. 2.—Six-day-old seedlings from seeds treated as follows: *A*, no treatment; *B*, *C*, 10,000 r, then hydrated for 30 minutes in the presence of oxygen (*B*) and nitrogen (*C*); *D*, *E*, 10,000 r, then hydrated for 240 minutes in the presence of oxygen (*D*) and nitrogen (*E*).

The reduced injury observed in the irradiated seedlings that developed from seeds hydrated in the presence of nitrogen was also obtained when seeds were hydrated in the presence of either hydrogen or helium. Thus the absence of oxygen rather than the presence of nitrogen is the point of significance in these studies.

Embryo Water Content.—Dormant seeds of barley having embryo water contents approximating 4 and 16 per cent showed a striking difference in the manner in which they responded to postradiation hydration in the presence of oxygen and nitrogen (Fig. 3). The seeds with the lowest water content incurred much greater injury by

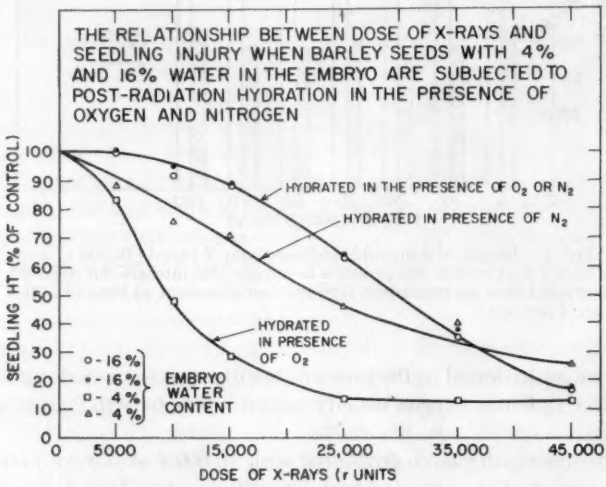

FIG. 3.—The relationship between dose of X-rays and seedling injury when barley seeds with 4 and 16 per cent water in the embryo are subjected to postradiation hydration in the presence of oxygen and nitrogen.

hydration in the presence of oxygen at all doses of radiation than did seeds having the same embryo water content but hydrated in the presence of nitrogen. This difference was in contrast to that obtained in the seed having the highest embryo water content where it was apparent that the conditions of hydration were ineffective in modifying radiation-initiated injury. It should be mentioned in connection with Figure 3 that, irrespective of the dose of X-rays given to seeds, they will always initiate germination and develop a certain minimum extent. This accounts for the apparent equivalence in injury evidenced by doses of 25,000, 35,000, and 45,000 r given to dry seeds that were subsequently hydrated in the presence of oxygen.

A comparison of the dose-height curves (Fig. 3) for seeds with embryo water contents of 4 per cent (hydrated in the presence of nitrogen) and 16 per cent (hydrated in the presence of either oxygen or nitrogen) showed an interesting relationship. At the lower doses of X-rays, posthydration of the dry seeds in the presence of nitrogen resulted in distinctly greater injury than in the case where seeds with an embryo water content of 16 per cent were hydrated in the presence of either of the two gases. However, as the dose increased, there was a gradual convergence of these two curves to a point at a barely sublethal dose where they actually crossed over. The validity

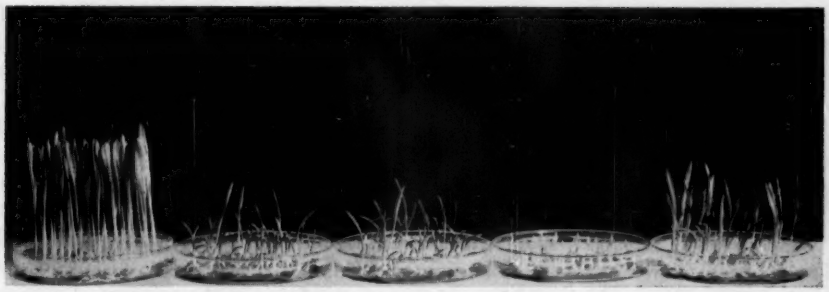

FIG. 4.—Six-day-old seedlings from seeds treated as follows: *A*, no treatment; *B*, *C*, 16 per cent embryo water content subjected to 45,000 r and hydrated 45 minutes in the presence of oxygen (*B*) and nitrogen (*C*); *D*, *E*, 4 per cent embryo water content subjected to 45,000 r and hydrated 45 minutes in presence of oxygen (*D*) and nitrogen (*E*).

of this observation has been substantiated by obtaining the same result in each of three experiments (cf. Fig. 4).

Ion Density.—It has been repeatedly demonstrated that biological systems are generally more sensitive to ionizing radiation when irradiated in the presence of oxygen than when under anoxic conditions.[3] From this report one can draw two general conclusions: (1) that the amount of oxygen available "during" irradiation is the significant fact; (2) that, as the ion density of radiations increases, there is a corresponding reduction in the sensitivity of organisms to the presence of oxygen during bombardment. Clearly, the data presented showing a posttreatment influence of oxygen on the radiosensitivity of barley seeds are at variance with the first conclusion. The question remained as to whether or not they would be at variance with the second conclusion. That is, would seeds subjected to radiations with a high ion density show similar postradiation sensitivity to hydration in the presence of oxygen.

To test this hypothesis, both dry and wet dormant seeds were irradiated in a number of different experiments with either fast or slow neutrons in the Brookhaven Nuclear Reactor and then immediately hydrated in the presence of either oxygen or nitrogen. These experiments showed conclusively that seeds subjected to both

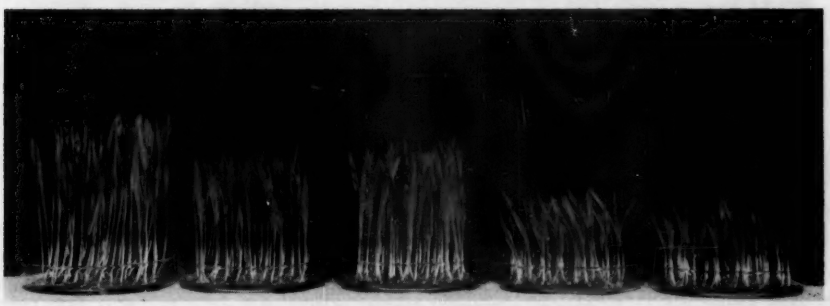

FIG. 5.—Six-day-old seedlings from seeds with a 4 per cent embryo water content treated as follows: *A*, no treatment; *B*, *C*, 30 minutes fast neutrons plus 45 minutes hydration in the presence of oxygen (*B*) and nitrogen (*C*); *D*, *E*, 60 minutes treatment with fast neutrons plus 45 minutes hydration in the presence of oxygen (*D*) and nitrogen (*E*).

sources of neutron radiation were not appreciably influenced by the conditions of postradiation hydration (Fig. 5). The implications of these experiments concerning the manner in which oxygen enhances radiation-initiated biological injury will be presented in the discussion.

Stability of the Observed Modifications.—One of the many questions that have arisen concerning these studies is the relative stability of the changes that can be induced in dry seeds by postradiation hydration with oxygen and nitrogen. For example, after hydration for 45 minutes in the presence of nitrogen, could seeds that were dry when X-rayed then be hydrated further in the presence of oxygen and still evidence an increase in radiation-initiated injury? To determine this point, X-rayed dry seeds were hydrated for various periods of time in the presence of oxygen and then hydrated for an equal time in the presence of nitrogen. A reciprocal series was also run in which initial hydration in the presence of nitrogen was followed by oxygen. The data showed that hydration in the presence of oxygen for any of the time periods used was not modifiable by subsequent hydration in the presence of nitrogen. On the other hand, initial hydration in the presence of nitrogen did not provide complete protection against subsequent hydration in the presence of oxygen. However, for the longer periods of initial hydration in the presence of nitrogen, i.e., from 30 minutes to 3 hours, the extent of the modification that was obtained from subsequent hydration in the presence of oxygen slowly diminished. The data are too unwieldy to warrant detailed presentation. However, a photograph of seedling development for one interval of 45 minutes is presented to illustrate the point in question (Fig. 6).

FIG. 6.—Six-day-old seedlings from seeds with a 4 per cent embryo water content that were treated as follows: *A*, no treatment; *B*, *C*, 15,000 r plus 45 minutes hydration in the presence of oxygen (*B*) and nitrogen (*C*); *D*, 15,000 r plus 45 minutes hydration in the presence of oxygen followed by 45 minutes hydration in the presence of nitrogen; *E*, 15,000 r plus 45 minutes hydration in the presence of nitrogen followed by 45 minutes hydration in the presence of oxygen.

DISCUSSION

Alper[4] has indicated that the likelihood of demonstrating a postradiation influence of oxygen on biological systems is remote because the life of ions induced by radiation is measured in milliseconds. Presumably, such a demonstration could occur only if the system had some unique method of storing ions or radiation-induced lesions that are sensitive to oxidation. If one takes the position that ion storage is unlikely, then the alternate explanation seems worthy of exploration.

It is considered significant that a postradiation effect of oxygen was not demonstrable on seeds with an embryo water content of about 16 per cent but only on seeds with an abnormally low embryo water content of about 4 per cent. It has been established that there is a distinct difference in the respiratory activity of seeds with these two water contents (unpublished). Seeds with the highest embryo water content consume oxygen at an easily detectable rate, whereas the seeds with the lowest embryo water content consume barely detectable amounts of oxygen. It may be inferred from this that, in the seeds with the lowest water content, oxygen can readily diffuse to the nucleus, which is considered to be the site of the most significant injury, and therein enhance the degree of radiation-initiated modification. Conversely, in the seeds with the higher water content, the existing potential to consume oxygen in the cytoplasm may be sufficient to prevent diffusion to the cell nucleus, with the result that the nucleus is maintained under anaerobic conditions even during hydration in the presence of oxygen.

When the neutron data are considered in the light of the foregoing explanation, the question arises as to the reason why seeds with the low water content do not evidence the same postirradiation influence of oxygen as is observed with X-rays. The most plausible explanation appears to rest with the difference in the energy distribution within critical sites that results from the two sources of radiation. As previously mentioned,[2, 5] in barley seeds bombarded with neutrons, most of the energy is distributed in dense clusters. This is in contrast to the energy distribution in seeds treated with X-rays, where there is produced a relative abundance of sites that have been subjected to only one or a few ionizations within a critical volume. It is postulated that those sites receiving only one or a few ionizations are the ones that are sensitive to oxidation. Thus, whether or not events resulting from one or a few ionizations become manifest would be dependent upon two factors: (1) the relative abundance of such sites and (2) the diffusion of free oxygen to those sites. In the case of neutrons, such sites presumably would be too infrequent to produce a detectable modification in seedling growth rate, irrespective of whether the seed had a high or low embryo water content. In the case of X-rays the frequency of those sites should be of the same order of magnitude in both wet and dry seeds. However, because of the respiratory activity of the wet seeds, only in the case of the dry seeds would oxygen have the opportunity to reach the critical scene and modify it in such a fashion as to become manifest in reduced seedling growth rate.

It is apparent that the data presented support the view previously expressed,[6, 7] viz., that hydrogen peroxide and free radicals cannot account for significant radiation-induced injury to seeds. Apparently what is of concern is whether or not restoration of radiation-induced lesions to a stable state takes place before oxidation occurs at the site of the lesion. This viewpoint has been eloquently expressed by Alper.[4] However, she is of the opinion that proof of the interpretation is impossible because, in so short a time as a millisecond after passage of the particle through a critical molecule, the oxygen would be too late to take part in what she classes as a metionic reaction. As it has been clearly demonstrated herein that, under appropriate conditions with seeds, oxygen can take part in modifying a critical site following X-radiation, it is apparent that Alper's metionic stage does not apply in the present situation. The writers prefer to look upon the sensitive site as one that is

merely unstable in the chemical sense and subject to reaction with oxygen to produce biologically detectable events.

The above considerations should mean that X-raying seeds with a high water content would produce comparable types of detectable events, and of the same order of magnitude, as are obtained when either wet or dry seeds are bombarded with neutrons, i.e., those events resulting from densely ionizing particles such as electron tails in the case of X-rays. Unpublished experimental evidence supports this interpretation. Detailed studies recently completed at the university have shown that the frequencies of seedling mutations and reciprocal interchange chromosomes produced when seeds with a high embryo water content are subjected to X-rays approach that obtained when fast or slow neutrons are used as the source of radiation[5].

The phenomena reported logically raise the question as to whether postirradiation storage of X-rayed seeds in oxygen versus nitrogen at atmospheric pressure could accomplish the same result as hydration in the presence of oxygen versus nitrogen. Studies in this laboratory (unpublished) indicate that the answer to the question is negative and that storage in both gases results in an equivalent increase in injury. However, the much more extensive data of Nilan and co-workers[8] suggest that storing seeds in oxygen at atmospheric pressure for from 2 to 5 weeks following X-radiation increases the frequency of detectable chromosomal aberrations from a given dose of X-rays over the frequency obtained when seeds given the same dose are subsequently stored in a nitrogen atmosphere. Whether or not one accepts either of these two experiments as critical, it seems likely that hydration facilitates movement of oxygen to sensitive sites. Possibly this occurs as a result of an increase in the rate of diffusion of oxygen within hydrated seeds over that which occurs in dry ones.

In earlier work[7] it was indicated that there was an inverse relationship between the radiosensitivity of barley seeds and their water content. The present studies have shown that this situation obtains only when seeds are permitted to hydrate aerobically. Clearly, the inverse relationship reported does not stem from differential sensitivity of the seeds to X-radiation per se but rather to the presence of oxygen following bombardment.

SUMMARY

Seeds of Himalaya barley with either 4 or 16 per cent water in the embryo were subjected to various doses of X-radiation and then hydrated in the presence of either oxygen or nitrogen for different periods of time. Subsequently they were grown in Petri dishes for 6 days, at which time seedling height measurements were made. On the basis of these measurements, the seeds with the lowest water content at the time of irradiation evidenced a striking sensitivity to postirradiation hydration in the presence of oxygen. In contrast to this, the seeds with the highest water content evidenced no injury modification that could be attributed to the conditions of hydration. Seeds with the same two water content levels that were subjected to thermal and fast neutron bombardment did not show postirradiation sensitivity to aerobic and anaerobic hydration.

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† Contribution from the U.S. Department of Agriculture, Field Crops Research Branch, ARS, and the University of Minnesota. Paper No. 3716, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[1] H. J. Curtis *et al.*, *Nucleonics*, **14**, 26–29, 1956.
[2] R. S. Caldecott, *Ann. N.Y. Acad. Sci.*, **59**, 514–35, 1955.
[3] L. H. Gray, *Radiation Research*, **1**, 189–213, 1954.
[4] T. Alper, *Radiation Research*, **5**, 573–86, 1956.
[5] R. S. Caldecott, *et al.*, *Genetics*, **39**, 240–59, 1954.
[6] R. S. Caldecott, *Radiation Research*, **2**, 339–50, 1955.
[7] R. S. Caldecott, *ibid.*, **3**, 316–30, 1955.
[8] J. D. Adams, *et al.*, *Northwest Sci.*, **29**, 101–8, 1955.

TRANSFORMATION GROUPS WITH TWO CLASSES OF ORBITS

BY ARMAND BOREL

INSTITUTE FOR ADVANCED STUDY, PRINCETON, NEW JERSEY

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G will be a connected compact Lie group and X a locally compact space on which G acts continuously and effectively; G_x is the isotropy group of $x \in X$, i.e., the subgroup of elements in G leaving x fixed. When G_x and G_y ($x, y \in X$) are conjugate by an inner automorphism of G, x, and y, or their orbits, are said to be in the same class. This note gives some results concerning mainly transformation groups with one or two classes of isotropy groups. The detailed proofs will appear elsewhere.

1. *Notation.*—G/U is the space of left cosets of G modulo a closed subgroup U, and N_U is the normalizer of U in G; $\boldsymbol{U}$, $\boldsymbol{V}$ will be classes of conjugate subgroups in G, and we write $\boldsymbol{U} < \boldsymbol{V}$ if every element of $\boldsymbol{U}$ is contained in some element of $\boldsymbol{V}$; $X_{\boldsymbol{U}}$ is the subspace of X consisting of the points with isotropy group in $\boldsymbol{U}$, and $F_U(U \in \boldsymbol{U})$ is the set of fixed points of U in $X_{\boldsymbol{U}}$.

An *equivariant* map $f: X \to Y$, where Y is a space also operated upon by G, is a continuous map such that $f(g.x) = g.f(x) (x \in X, g \in G)$.

2. *Two Fiberings of $X_{\boldsymbol{U}}$.*—Using the operations of N_U on $X_{\boldsymbol{U}}(U \in \boldsymbol{U})$ and the right translations of N_U in G, we see immediately that F_U and G/U are principal bundles for the group N_U/U; these operations are meant to define the structural group in the following:

PROPOSITION 2.1. *The subspace $X_{\boldsymbol{U}}$ admits two locally trivial fiberings ξ_1, ξ_2 with structural group N_U/U. The fibering ξ_1 (resp. ξ_2) has G/N_U (resp. $X_{\boldsymbol{U}}/G$), as base, and its fibers are the subspaces $F_{U'}(U' \in \boldsymbol{U})$ (resp. the orbits of G in $X_{\boldsymbol{U}}$).*

We say that $\boldsymbol{U}$ is the biggest (resp. smallest) class if $\boldsymbol{U} > \boldsymbol{V}$, (resp. $\boldsymbol{U} < \boldsymbol{V}$) for every class of isotropy groups of G in X; there is clearly at most one such class. The smallest one always exists when X is a connected manifold.[1]

PROPOSITION 2.2. *Assume that G operating on X has a smallest class $\boldsymbol{U}$ and a biggest class $\boldsymbol{V}$ of isotropy groups and that every element of $\boldsymbol{U}$ is contained in exactly*

one element of $\boldsymbol{V}$. *Then there is an equivariant map* α: $X \to G/N_V(V \in \boldsymbol{V})$ *whose restriction to* X_V *is the projection in the fibering* ξ_1 *of Proposition 2.1.*

If $N_v = \boldsymbol{V}$, it follows that α_0: $\pi_1(X) \to \pi_1(G/V)$ is surjective. From results of Smith and from known properties of maximal tori in G, we deduce

COROLLARY 2.3. *If the assumptions of Proposition 2.2 are satisfied and if* X *is homeomorphic to Euclidean n-space, then* G *has a fixed point.*

3. *One Class of Orbits.*—S_n denotes the n-dimensional sphere; $U(1)$ (resp. $Sp(1)$) the group of complex (resp. quaternionic) numbers of norm 1; $SO(3)$ the orthogonal unimodular group in three real variables.

THEOREM 3.1. *Assume that* $X = S_n$ *and that* G *has only one class of orbits on* X. *Then, if* G *is not transitive, it is isomorphic to* $U(1)$ *or* $Sp(1)$, *the isotropy groups are reduced to the identity, and* $n + 1$ *is divisible by 2 or 4.*

Examples of this theorem are, of course, the Hopf fiberings over the complex or quaternionic projective spaces; we do not know whether these are the only ones, up to equivariant homeomorphisms. To prove the theorem, one remarks first that an orbit has the real cohomology of S_k (k odd) which follows, e.g., from a result of Spanier-Whitehead;[2] then, using the Gysin sequence and Proposition 2.1, one shows that an isotropy group U is invariant in G, whence $U = (e)$ and rank $G = 1$; finally, $SO(3)$ is excluded by the consideration of its diagonal matrices.

4. *Two Classes of Orbits.*—$H^*(X, L)$ is the Alexander-Spanier cohomology ring of X with coefficients in the commutative ring L. We say that X is clc (cohomologically locally connected) over L if, for each $x \in X$, integer $m \geqslant 0$, and neighborhood W of x, there exists a neighborhood S of x in W such that the natural map of $H^m(W, L)$ in $H^m(S, L)$ has zero image.[3] In Theorem 4.1 below, the notion of generalized manifold may be taken in the sense of Smith[4] or Wilder;[5] we refer to another paper[6] for a discussion of these from the point of view of Alexander-Spanier cohomology and sheaf theory.

THEOREM 4.1. *Let the one point compactification of* X *be clc over the integers, have the integral cohomology of* S_n, *and for each prime* p *be a generalized orientable n-manifold over the integers mod* p. *If* G *has exactly two classes of orbits on* X, *then one consists of fixed points.*

The proof is by induction on dim G. Let $\boldsymbol{U}$ be the smaller class of isotropy groups. When $U \in \boldsymbol{U}$ is infinite, one considers the subspace Y of fixed points of a maximal torus T of U, acted upon by the quotient H/T by T of its centralizer in G, and, using notably results of Smith and Floyd,[7] one shows that H/T and Y satisfy the assumptions of Theorem 4.1; the existence of fixed points follows then from Proposition 2.2 and the induction assumption. When U is finite, one proves, by means of transgression theorems of an earlier paper,[8] that G is three-dimensional. The remainder of the proof consists in a detailed discussion in cohomology mod 2, involving in particular the subgroup of diagonal matrices of $SO(3)$ and its normalizer.

THEOREM 4.2. *Let* X *be a connected n-dimensional differentiable manifold on which* G *operates differentiably. Assume that the subspace* F *of fixed points is not empty and that* G *has one class of orbits in* $X - F$. *Then the orbits in* $X - F$ *are homeomorphic to* S_m. *If* $m = n - \dim F - 1$, *then* $G = U(1)$ *and the isotropy groups in* $X - F$ *are reduced to the identity. If* X *has the integral cohomology of the Euclidean n-space so has* F.

Let x be a fixed point. Then, by a well-known result of Bochner, the action around x is linear in suitable local coordinates. The theorem follows then from Theorem 3.1 and a spectral sequence argument.

[1] This follows from Theorem 1 in D. Montgomery, H. Samelson, and L. Zippin, "Singular Points of a Compact Transformation Group," *Ann. Math.*, **63**, 1–9, 1956.

[2] E. Spanier and H. H. C. Whitehead, "On Fibre Spaces in Which the Fibre Is Contractible," *Comm. Math. Helv.*, **29**, 1–8, 1954.

[3] In dimension zero, reduced cohomology groups are used.

[4] P. A. Smith, "Transformations of Finite Period. II," *Ann. Math.*, **40**, 690–711, 1939.

[5] R. L. Wilder, *The Topology of Manifolds* ("Am. Math. Soc. Colloquium Publs.," No. 32 [New York, 1949]).

[6] A. Borel, "The Poincaré Duality in Generalized Manifolds," to appear in *Mich. J. Math.*

[7] E. E. Floyd, "Fixed Point Sets of Compact Abelian Lie Groups of Transformations," *Am. Math.*, **66**, 30–35, 1957.

[8] A. Borel, *Ann. Math.*, **57**, 115–207, 1953.

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*ON UNITARY REPRESENTATIONS OF NILPOTENT LIE GROUPS**

BY JACQUES DIXMIER

INSTITUT HENRI POINCARE

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Let G be a real Lie group of dimension n; $\mathfrak{g}$ its Lie algebra; $\mathfrak{U}(\mathfrak{g})$ the enveloping algebra of $\mathfrak{g}$; $\mathfrak{Z}(\mathfrak{g})$ the center of $\mathfrak{U}(\mathfrak{g})$; A the antiautomorphism of $\mathfrak{U}(\mathfrak{g})$ which transforms $x_1x_2 \ldots x_r$ (where $x_1, \ldots, x_r \in \mathfrak{g}$) into $(-1)^r x_r x_{r-1} \ldots x_1$. Let $\mathfrak{Z}^+(\mathfrak{g})$ (resp. $\mathfrak{Z}^-(\mathfrak{g})$) be the set of all elements in $\mathfrak{Z}(\mathfrak{g})$ such that $Aa = a$ (resp. $Aa = -a$). A hermitian character of $\mathfrak{Z}(\mathfrak{g})$ is a homomorphism χ of $\mathfrak{Z}(\mathfrak{g})$ into C^1 such that $\chi(a)$ is real for $a \in \mathfrak{Z}^+(\mathfrak{g})$ and purely imaginary for $a \in \mathfrak{Z}^-(\mathfrak{g})$. Let H be a Hilbert space; U a (strongly continuous) unitary irreducible representation of G in H; H_1 the vector subspace of H consisting of the infinitely differentiable elements for U. One knows that U defines a representation (which we shall denote still by U) of $\mathfrak{U}(\mathfrak{g})$ in H_1 and that $U(a)$ is a scalar operator $\chi(a) \cdot 1$ if $a \in \mathfrak{Z}(\mathfrak{g})$.[2] One says that the mapping $a \to \chi(a)$, which is a hermitian character of $\mathfrak{Z}(\mathfrak{g})$, is the character of U. This character does not change if one replaces U by a (unitarily) equivalent representation. Even if G is solvable, it is completely false that χ determines U up to a unitary equivalence. But one has the following theorem:

THEOREM 1. *If G is nilpotent and simply connected, there exists in $\mathfrak{Z}(\mathfrak{g})$ an element $d \neq 0$ such that every hermitian character χ of $\mathfrak{Z}(\mathfrak{g})$ with $\chi(d) \neq 0$ corresponds to one and (up to a unitary equivalence) only one unitary irreducible representation of G.*

Moreover, there exist elements $c_1, \ldots, c_q$ of $\mathfrak{Z}^-(\mathfrak{g})$, and a polynomial Q in q variables with real coefficients, such that the mapping $\chi \to (\chi(c_1), \ldots, \chi(c_q))$ is a one-to-one mapping of the set of hermitian characters of $\mathfrak{Z}(\mathfrak{g})$ different from zero at d, onto the set of systems $(\lambda_1, \ldots, \lambda_q)$ of purely imaginary numbers with $Q(\lambda_1, \ldots, \lambda_q) \neq 0$. So one sees that the unitary irreducible representations considered in Theorem 1 are indexed by q real parameters not annihilating a certain polynomial.

An element d of $\mathfrak{Z}(\mathfrak{g})$ possessing the properties of Theorem 1 will be called a

classifying element. We shall denote by $\Lambda_d(\mathfrak{g})$ the set of hermitian characters χ of $\mathfrak{Z}(\mathfrak{g})$ such that $\chi(d) \neq 0$. As we have just seen, $\Lambda_d(\mathfrak{g})$ can be identified with a set Ω of an affine real space of dimension q, Ω being open in the Zariski topology.

For Theorem 1, some algebraic results are necessary. First, the field of fractions of $\mathfrak{Z}(\mathfrak{g})$ is a purely transcendental extension of R; its degree of transcendence is $n - 2p$, p being an integer called the *commutativity deviation* of $\mathfrak{g}$. Let $\mathfrak{g}'$ be an ideal of codimension 1 in $\mathfrak{g}$; if $\mathfrak{Z}(\mathfrak{g}) \subset \mathfrak{U}(\mathfrak{g}')$, then $\mathfrak{Z}(\mathfrak{g}) \supset \mathfrak{Z}(\mathfrak{g}')$, and the degree of transcendence of $\mathfrak{Z}(\mathfrak{g})$ over $\mathfrak{Z}(\mathfrak{g}')$ is 1; if $\mathfrak{Z}(\mathfrak{g}) \subset \mathfrak{U}(\mathfrak{g}')$, then $\mathfrak{Z}(\mathfrak{g}) \subset \mathfrak{Z}(\mathfrak{g}')$, and the degree of transcendence of $\mathfrak{Z}(\mathfrak{g}')$ over $\mathfrak{Z}(\mathfrak{g})$ is 1.

THEOREM 2. *Suppose G nilpotent and simply connected. Let p be the commutativity deviation of $\mathfrak{g}$, H the Hilbert space $L_C^2(R^p)$, H_1 the subspace of H consisting of infinitely differentiable and rapidly decreasing functions on R^p. Let D_j and M_j be the operators, defined in H_1, of differentiation with respect to the j^{th} variable and of multiplication by the j^{th} variable. Let A be the operator algebra in H generated by 1, $M_1, \ldots, M_p, D_1, \ldots, D_p$. There exist a classifying element d of $\mathfrak{Z}(\mathfrak{g})$ and, for all $\chi \in \Lambda_d(\mathfrak{g})$, a unitary irreducible representation U_χ of G in H, of character χ, with the following properties:*

(i) *The elements of H which are infinitely differentiable for U_x are the elements of H_1.*

(ii) *The algebra of operators $U_\chi(e)$, where e runs over $\mathfrak{U}(\mathfrak{g})$, is A.*

(iii) *For all $e \in \mathfrak{U}(\mathfrak{g})$, there exist elements $T_1, \ldots, T_r$ of A, elements $e_1, \ldots, e_r$ of $\mathfrak{Z}(\mathfrak{g})$, and an integer $s \geq 0$, independent of χ, such that $U_\chi(e) = \chi(d)^{-s}[\chi(e_1)T_1 + \ldots + \chi(e_r)T_r]$.*

For $a \in \mathfrak{U}(\mathfrak{g})$, the mapping $\chi \to U_\chi(a)$ is the Fourier transform, defined in $\Lambda_d(\mathfrak{g}) = \Omega$, of the distribution a over G. According to Theorem 2, it is a regular function on the variety Ω. If μ is a bounded measure over G, the mapping $\chi \to U_\chi(\mu) = \int U_\chi(g)d\mu(g)$ of Ω in the space of continuous operators in H is the Fourier transform of μ; one can prove that it is strongly continuous and that it tends uniformly toward 0 at infinity if μ is defined by a function of $L^1(G)$.

THEOREM 3. *There exists a positive measure ν on Ω, defined by a regular differential form of degree q, such that, for all $f \in L_C^1(G) \cap L_C^2(G)$, the function $\chi \to Tr[U_\chi(f)^*U_\chi(f)]$ over Ω is ν-integrable, with*

$$\int_G |f(g)|^2 dg = \int_\Omega Tr[U_\chi(f)^*U_\chi(f)]d\nu(\chi).$$

We can also decompose the two-sided regular representation of G in a continuous direct sum (over Ω) of the representations $1 \otimes U_\chi$ and $U_\chi \otimes 1$ in $L_C^2(R^p) \otimes L_C^2(R^p) = L_C^2(R^{2p})$.

It is known that the Plancherel formula involves, in general, factor representations. In our case, one can prove that nilpotent (and even algebraic) Lie groups are of type I; so it is not surprising that irreducible representations are sufficient.

* This research was supported in part by United States Air Force under Contract No. AF 18(600)-1383 monitored by AF Office of Scientific Research, Air Research and Development Command.

[1] We denote by C the complex field, by R the real field.

[2] I. E. Segal, *Proc. Am. Math. Soc.*, **3**, 13–15, 1952.

PRODUCTS IN HOMOTOPY AND HOMOLOGY GROUPS

By J. Dugundji

UNIVERSITY OF SOUTHERN CALIFORNIA

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1. *Introduction.*—Many properties of the Whitehead products (such as their vanishing in H-spaces) can very easily be obtained by developing an antecedent product (the principal pairing) which gives rise to it. This new product also generates several others which have a marked utility. In this note, these products and corresponding ones for singular integral homology are defined; applications will be given elsewhere. A portion of this work was done in collaboration with W. Hurewicz.

2. *The Reflectant.*—Let Y be a Hausdorff space, and $y_0 \in Y$. In $Y \times Y$ identify each point (y, y_0) with (y_0, y). The resulting space, called the *reflectant* of Y based on y_0, is written $R(Y, y_0)$; μ: $Y \times Y \to R(Y, y_0)$ denotes the identification map, and Y is identified with its homeomorph, $(Y, y_0) \subset R(Y, y_0)$. The homotopy type of $R(Y, y_0)$ depends on y_0 in general; however,

2.1. *If Y is an ANR, then $R(Y, y_0)$ and $R(Y, y_0')$ belong to a common homotopy type whenever y_0, y_0' are in a common arc component.*

The reflectant is related to H-structures; for

2.2 *Y carries an H-structure with unit*[1] *y_0 if and only if it is a retract of $R(Y, y_0)$.*

One uses the formula $y \vee y' = r\mu(y, y')$, where r: $R(Y, y_0) \to Y$ is a retraction.

We remark that the condition that the H-structure have a unit is no restriction[2] if Y is an arc-connected ANR.

3. *The Principal Pairing.*—This pairs $\pi_p(Y)$ and $\pi_q(Y)$ to $\pi_{p+q}(R, Y)$. Let $\alpha_p \in \pi_p(Y, y_0)$, $\alpha_q \in \pi_q(Y, y_0)$ be represented by maps f: $(I^p, \dot{I}^p) \to (Y, y_0)$, g: $(I^q, \dot{I}^q) \to (Y, y_0)$, respectively; using the origin as base point on the cubes, the map

$$f \cdot g(u, v) = \mu(f(u), g(v)), \qquad (u, v) \in I^p \times I^q,$$

represents an element $\alpha_p \cdot \alpha_q \in \pi_{p+q}(R(Y, y_0), Y, y_0)$ depending only on α_p and α_q and not on the representatives chosen.

Let ρ be the reflection $\rho(y, y') = (y', y)$; $\rho \mid Y$ is the identity.

3.1. (*a*) *For $p > 1$, $(\alpha_p + \beta_p) \cdot \alpha_q = \alpha_p \cdot \alpha_q + \beta_p \cdot \alpha_q$;*
(*b*) $\rho(\alpha_p \cdot \alpha_q) = (-1)^{pq}\, \alpha_q \cdot \alpha_p$.

A pairing of the singular integral homologies $H_p(Y)$ and $H_q(Y)$ to $H_{q+q}(R)$ (not the relative group) related to the principal pairing is obtained by setting

$$\xi_p \cdot \xi_q = \mu_+(\xi_p \times \xi_q), \qquad \xi_p \in H_p(Y), \quad \xi_q \in H_q(Y).$$

It is clear that $\rho(\xi_p \cdot \xi_q) = (-1)^{pq}\, \xi_q \cdot \xi_p$. Furthermore,

3.2. *Let h be the Hurewicz homomorphism of the homotopy groups of a space into its homology groups, and j: $H_n(R) \to H_n(R, Y)$ the injection. Then $h(\alpha_p \cdot \alpha_q) = j(h(\alpha_p) \cdot h(\alpha_q))$ if $p, q \geq 2$.*

4. *Whitehead Product.*—Let ∂: $\pi_n(R(Y, y_0), Y, y_0) \to \pi_{n-1}(Y, y_0)$ be the boundary homomorphism.

4.1. $\partial(\alpha_p \cdot \alpha_q)$ *is the Whitehead product* $[\alpha_p, \alpha_q]$.

It is immediate that all Whitehead products in any arc-connected H-space are zero. For any H-space can be imbedded as a deformation retract of an H-space with unit,[3] and, by 2.2, $\partial\pi_n(R, Y, y_0) = 0$. The further consequence that S^n is an H-space if and only if $[i, i] = 0$, i the generator of $\pi_n(S^n)$, is now trivial.

5. *Separation Pairing.*—This pairs $\pi_q(Y)$ and $\pi_q(Y)$ to $\pi_{p+q}(R)$. The identity map 1 and the reflection ρ both agree on Y, so there is a separation homomorphism[4] $\Delta(1, \rho)$: $\pi_n(R, Y, y_0) \to \pi_n(R, y_0)$. The separation pairings are defined by

$$\alpha_p \circ \alpha_q = \Delta(1, \rho)\ (\alpha_p \cdot \alpha_q),$$
$$\xi_p \circ \xi_q = \xi_p \cdot \xi_q - \rho(\xi_p \cdot \xi_q) \equiv \xi_p \cdot \xi_q - (-1)^{pq}\ \xi_q \cdot \xi_p.$$

Using 3.1 and 3.2, one has

5.1. $\alpha_p \circ \alpha_q = (-1)^{pq+1}\ \alpha_q \circ \alpha_p$; $h(\alpha_p \circ \alpha_q) = h(\alpha_p) \circ h(\alpha_q)$ *if* $p, q \geq 2$.

6. *Composition Pairing.*—Whenever Y has an H-structure with unit y_0, the principal pairing generates one of $\pi_p(Y)$, $\pi_q(Y)$ to $\pi_{p+q}(Y)$ (and also for homology) by setting

$$\{\alpha_p, \alpha_q\} = r(\alpha_p \circ \alpha_q),$$
$$\{\xi_p, \xi_q\} = r(\xi_p \circ \xi_q).$$

It is obvious that $r(\xi_p \cdot \xi_q)$ is precisely the Pontrjagin product $\xi_p * \xi_q$, so that $\{\xi_p, \xi_q\} = \xi_p * \xi_q - (-1)^{pq}\ \xi_q * \xi_p$.

6.1. $h\{\alpha_p, \alpha_q\} = \{h(\alpha_p), h(\alpha_q)\}$, $\quad p, q \geq 2$.

6.2. *If the H-structure on Y is a group structure, and if f and g represent* α_p *and* α_q, *respectively, then* $\{\alpha_p, \alpha_q\}$ *is represented by* $f(u)g(v)f(u)^{-1}g(v)^{-1}$: $(I^{p+q}, \dot{I}^{p+q}) \to (Y, y_0)$.

The composition pairing can also be defined when the H-structure in Y does not have a unit and 6.1 still holds; a modification of the result in 6.2 is valid in H-structures with inverses.[5] With this generalization, if τ is the natural isomorphism τ: $\pi_n(Y) \approx \pi_{n-1}(\Omega)$, Ω the loop space of Y based at y_0, then the Whitehead and composition products are related by

6.3. *If* $p, q, \geq 2$, *then* $\tau[\alpha_p, \alpha_q] = (-1)^{p-1}\{\tau\alpha_p, \tau\alpha_q\}$.

[1] That is, $y_0 \vee y = y \vee y_0 = y$ for every $y \in Y$.

[2] If Y is an arc-connected ANR and F: $Y \times Y \to Y$ is the composition map for any H-structure on Y, then for any given $y_0 \in Y$ there is an F': $Y \times Y \to Y$ with $F'(y, y_0) = F'(y_0, y) = y$ and $F' \simeq F$.

[3] The H-structure on the containing space is an extension of that on Y, and the deformation retraction is an H-isomorphism.

[4] J. H. C. Whitehead, *Ann. Math.*, **52**, 108, 1950.

[5] That is, there is a map φ: $Y \to Y$ with $y \to y \vee \varphi(y)$ nullhomotopic; $\varphi(y)$ is written y^{-1}.

THE MULTIDIMENSIONAL PREDICTION PROBLEM*

M. ROSENBLATT

INDIANA UNIVERSITY

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A simple proof of an extension of one of Wiener's results on prediction is given. The proof reduces consideration of the multidimensional problem to the one-dimensional problem.

Suppose

$$x_t = (x_t^{(1)}, \ldots, x_t^{(n)})' \qquad t = 0, \pm 1, \ldots$$

(A' is the transpose conjugate of the matrix A) is a weakly stationary n-vector-valued process. For convenience we assume that $Ex_t \equiv 0$. Since x_t is weakly stationary, the covariance matrix $r_{t,\tau} = Ex_t x_\tau' = r_{t-\tau}$ exists and depends only on $t - \tau$. The covariance matrices r_t are the Fourier-Stieltjes coefficients of a non-decreasing function F of bounded variation,

$$r_t = \int_{-\pi}^{\pi} e^{it\lambda}\, dF(\lambda).$$

A case of especial interest to us is that in which F is absolutely continuous $F'(\lambda) = f(\lambda)$. Then

$$r_t = \int_{-\pi}^{\pi} e^{it\lambda} f(\lambda) d\lambda.$$

The function f is called the "spectral density function" of the process and is a non-negative definite matrix-valued function of λ.

Let us now consider the prediction problem. Suppose we wish to predict x_t by a linear expression in terms of the past, i.e., $x_{t-1}, x_{t-2}, \ldots$. Call the predictor x_t^*. The predictor is the best linear predictor in the sense of mean square if it minimizes

$$E(x_t - x_t^*)(x_t - x_t^*)'. \tag{1}$$

It is clear that x_t^* is the projection of x_t on the closed linear manifold M_{t-1} generated by the components of $x_{t-1}, x_{t-2}, \ldots$. Paralleling the one-dimensional context, we call x_t purely deterministic if $x_t = x_t^*$ and x_t purely nondeterministic if the projection of on the infinite past ΠM_t is the null vector.

It is of especial interest to be able to obtain a characterization of the class of purely nondeterministic processes in terms of the spectrum of the process. A partial result in this direction has been given by N. Wiener.[1] This result characterizes the class of purely nondeterministic processes with nonsingular prediction error (1). First, consider predicting x_t by a linear expression in $x_{t-1}, \ldots, x_{t-m}$, i.e.,

$$x_t^* = \sum_{k=1}^{m} c_k^{(m)} x_{t-k},$$

where the $c_k^{(m)}$ are $n \times n$ matrices. The prediction error is

$$E_m = \min_{c^{(m)}} E(x_t - x_t^*)(x_t - x_t^*)'$$

$$= \min_{c^{(m)}} \int_{-\pi}^{\pi} [I - c^{(m)}(e^{-i\lambda})] f(\lambda) [I - c^{(m)}(e^{-i\lambda})]' d\lambda,$$

where

$$c^{(m)}(e^{-i\lambda}) = \sum_1^m c_k^{(m)} e^{-ik\lambda}.$$

The sequence of matrices $\{E_m\}$ is a nonincreasing sequence of matrices $\ldots \geqslant E_m \geqslant E_{m+1} \geqslant \ldots$ and there is a well-defined limiting matrix $E_\infty = \lim_{m\to\infty} E_m$ (see Doob[2]). Doob has shown that if E_∞ is positive definite, $f(\lambda)$ has a representation

$$f(\lambda) = (\sum_{j=0}^{\infty} c_j e^{-ij\lambda})(\sum_{j=0}^{\infty} c_j e^{-ij\lambda})' \tag{2}$$

where $\Sigma\, c_j c_j' < \infty$ and $c_0 = E_\infty^{1/2}$ (the positive definite square root). Further, the function $c(z) = \sum_{j=0}^{\infty} c_j z^j$ entering into representation (2) is analytic when $|z| < 1$ and can be chosen so that $|c(z)|$ has no zeros in $|z| < 1$ ($|A|$ denotes the determinant of A).

Now $|f(\lambda)|^{1/n} \epsilon L$. For, if $g_1(\lambda), \ldots, g_n(\lambda)$ are the eigenvalues of $f(\lambda)$.

$$|f(\lambda)| = \prod_1^n g_i(\lambda)$$

and the $g_i(\lambda)$ $i = 1, \ldots, n$ are in L. But then

$$\int_{-\pi}^{\pi} |f(\lambda)|^{1/n} d\lambda = \int_{-\pi}^{\pi} [\prod_1^n g_i(\lambda)]^{1/n} d\lambda$$

$$\leq [\int_{-\pi}^{\pi} g_1(\lambda) d\lambda]^{1/n} [\int_{-\pi}^{\pi} (\prod_2^n g_i(\lambda))^{1/n-1} d\lambda]^{n-1/n} \leq \ldots \leq \prod_1^n [\int_{-\pi}^{\pi} g_i(\lambda) d\lambda]^{1/n}.$$

Thus $|f(\lambda)|^{1/n}$ has the representation

$$|f(\lambda)|^{1/n} = |c(e^{-i\lambda})|^{1/n} |c(e^{-i\lambda})'|^{1/n},$$

where $|c(e^{-i\lambda})|^{1/n}$ is in L^2 and has a one-sided Fourier expansion. Further, we have assumed that $E_\infty = c_0^2$ is positive definite, so that $|E_\infty| = |c_0|^2 = |c(0)|^2 > 0$. However, this can be so only if $\log |f(\lambda)|^{1/n} = 1/n \log |f(\lambda)| \epsilon L$. We have the following result:

If x_t is a vector-valued weakly stationary process with an absolutely continuous spectrum and the one-step prediction error E_∞ is positive definite, then $\log |f(\lambda)| \epsilon L$ *and the determinant of the one-step prediction error is given by*

$$|E_\infty| = (2\pi)^n \exp\left\{\frac{1}{2\pi}\int_{-\pi}^{\pi} \log |f(\lambda)|\, d\lambda\right\}.$$

We would now like to show that *if* $\log |f(\lambda)| \epsilon L$, *the prediction error is positive definite.* It will be useful to have the following simple lemma.

LEMMA 1. *If A, B are nonnegative definite matrices then*

$$|A + B| \geqslant |A| + |B|.$$

Diagonalize the matrix A by the unitary matrix U so that

$$UAU' = \begin{pmatrix} \lambda_1 & & 0 \\ & \ddots & \\ 0 & & \lambda_n \end{pmatrix}.$$

Let $\{UBU'\}_{i_1 \ldots i_j}$ be the submatrix of UBU' obtained from UBU' by deleting the $i_1, \ldots, i_j$th rows and columns. Then

$$|A + B| = |U(A + B)U'|$$

$$= \lambda_1 \ldots \lambda_n + |UBU'| + \sum_{j=1}^{n-1} \sum_{i_1 \neq \cdots \neq i_j} \lambda_{i_1} \ldots \lambda_{i_j} |\{UBU'\}_{i_1 \ldots i_j}|$$

$$\geqslant \lambda_1 \ldots \lambda_n + |UBU'| = |A| + |B|.$$

The following remark is an immediate consequence of lemma 1.

LEMMA 2. *Let $g(\lambda)$ be an $n \times n$ non-negative definite matrix-valued function in L such that $|g(\lambda)|$ is integrable. Then*

$$\left| \int_{-\pi}^{\pi} g(\lambda) d\lambda \right| \geqslant \int_{-\pi}^{\pi} |g(\lambda)| d\lambda.$$

Now define the auxiliary non-negative definite matrix-valued function $f(\lambda)$ as follows:

$$f_-(\lambda) = \begin{cases} f(\lambda) & \text{if} \quad |f(\lambda)| \leq 1 \\ f(\lambda)/|f(\lambda)| & \text{if} \quad |f(\lambda)| > 1. \end{cases}$$

Clearly, $f(\lambda) \geqslant f_-(\lambda)$. Thus

$$E_m = \min_{c^{(m)}} \int_{-\pi}^{\pi} [I - c^{(m)}(e^{-i\lambda})] f(\lambda) [I - c^{(m)}(e^{-i\lambda})]' d\lambda$$

$$\geqslant \min_{c^{(m)}} \int_{-\pi}^{\pi} [I - c^{(m)}(e^{-i\lambda})] f_-(\lambda) [I - c^{(m)}(e^{-i\lambda})]' d\lambda$$

$$= E_m^-.$$

By lemma 2

$$|E_m^-| \geqslant \min_{c^{(m)}} \int_{-\pi}^{\pi} |I - c^{(m)}(e^{-i\lambda})| \, |f_-(\lambda)| \, |I - c^{(m)}(e^{-i\lambda})'| d\lambda.$$

On the right we have an ordinary one-dimensional approximation problem. The limit of the error on the right as $m \to \infty$ is

$$(2\pi)^n \exp\left\{ \frac{1}{2\pi} \int_{-\pi}^{\pi} \log |f_-(\lambda)| d\lambda \right\} > 0,$$

since $\log |f(\lambda)| \in L$. Thus

$$\lim_{m \to \infty} |E_m| = |E_\infty| > 0$$

and E_∞ is positive definite.

There are a few immediate consequences. First, *a necessary and sufficient condition that a non-negative definite $n \times n$ matrix-valued function $f(\lambda) \in L$ have a representation of the form* (2) *with c_0 nonsingular is that*

$$\log |f(\lambda)| \in L.$$

It is also clear that *a necessary and sufficient condition that a weakly stationary process x_t with spectral distribution function $F(\lambda)$ have a positive definite prediction error E_∞ is that*

$$\log |F'(\lambda)| \epsilon L.$$

The analogue of this result for a continuous-parameter weakly stationary process. is obtained by a small modification of the argument given above. *A necessary and sufficient condition that a continuous-parameter weakly stationary process with spectral distribution function $F(\lambda)$ have a positive definite prediction error is that*

$$\frac{\log |F'(\lambda)|}{1 + \lambda^2} \epsilon L.$$

It is now clear that there are a number of questions that ought to be cleared up. First of all, it is unpleasant and unnatural to insist on the error being nonsingular when getting conditions for a representation of the form (2). A necessary and sufficient condition for a representation of the form (2) in terms of the spectrum that does not make such a requirement ought to be obtained. A more difficult problem appears to be that of obtaining E_∞ in a natural and simple way in terms of the spectrum. It is easy, as we have seen, to obtain $|E_\infty|$ in terms of the spectrum, as this is a simple generalization of the one-dimensional result. But the problem of obtaining E_∞ in terms of the spectrum appears to be generally a noncommutative problem. There is also the question of answering the analogous problem in the infinite dimensional case in a neat way.

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[1] N. Wiener, "On the Factorization of Matrices," *Comm. Math. Helv.*, pp. 97–111, 1955.

[2] J. L. Doob, *Stochastic Processes* (New York: John Wiley & Sons, 1957).

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*UNIFORM CONVERGENCE FOR MONOTONE MAPPINGS**

By Gordon T. Whyburn

UNIVERSITY OF VIRGINIA

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1. *Introduction.*—The value and usefulness of uniform convergence in connection with a sequence of functions or mappings is accepted beyond question in any mathematical discussion, enabling one, as it does, to evaluate the effect on the limit function of the operation of so many processes or properties on the members of the sequence. Thus there is a sustained interest in conditions which may imply uniform convergence in settings of significance. A number of these are known in classical analysis. The theorems of Vitali and of Osgood are well-known cases in point. Extensions of these to a more general topological setting or even to the case of real functions seem to be rather sparse and limited. In this paper the object will be to outline some recent results and methods leading to existence conclusions for uniform convergence of sequences of mappings in a topological setting of interest and to indicate their relation and applicability to sequences of real functions in particular.

To illustrate our thinking here, we note first that there exists a sequence of

"square" mappings (i.e., mappings equivalent topologically to $w = z^2$) of a circle C onto itself which converges everywhere to the identity mapping but where the convergence is not uniform—as, indeed, it could not be by known theorems in topology. This can be effected on the circle C: $r = 1, 0 \leq \vartheta \leq 2\pi$ in the (r,ϑ) plane by defining, for each $n = 1, 2, \ldots$,

$$f_n(\vartheta) = e^{is(n,\vartheta)}$$

where

$$\begin{cases} s(n, \vartheta) = n\vartheta \text{ for } 0 \leq \vartheta \leq 2\pi/n \\ \qquad = (n\vartheta - 2\pi)/(n-1) \text{ for } 2\pi/n \leq \vartheta \leq 2\pi. \end{cases}$$

In contrast to this, a much more general theorem will be proved below, from which it follows that if X and Y are circles and the sequence $h_n(x)$ of homeomorphisms (or monotone mappings) of X onto Y converges everywhere on X to a mapping $f(x)$, then either f is constant or the convergence is uniform and f is monotone. Both cases mentioned in the conclusion here are realizable. For, letting both X and Y be the circle C used above and for each n letting $h_n(\vartheta)$ map the arc $0 \leq \vartheta \leq 2\pi/n$ of C topologically onto the arc $2\pi/n \leq \vartheta \leq 2\pi$ with ends fixed and the arc $2\pi/n \leq \vartheta \leq 2\pi$ likewise onto the arc $0 \leq \vartheta \leq 2\pi/n$, then $h_n(\vartheta)$ converges everywhere on C to the constant mapping $f(\vartheta) = 1$, but nonuniformly, of course.

The form of the theorem to be proved in this note is confined, for the sake of simplicity of statement, to the case where the domain space X of the mappings is a continuum (compact and connected) and where Y is cyclic and the mappings in the sequence are monotone and onto. "Cyclic" here means that no point of Y separates Y so that $Y - y$ is connected for each $y \in Y$. However, in later sections, statements of corresponding results in much less restricted cases will be given, together with a discussion of the applications of these results to real functions and to other simplified situations. In the final section a similar statement will be given for a result concerned with sequences of quasi-open mappings rather than monotone ones. In a later paper these results will be established in detail along with a further development of the ideas involved.

2. *The Compact Cyclic Case.*—All spaces used will be separable and metric. Distances will be denoted by $\rho(x,y)$ and diameters of sets by $\delta(N)$. The spherical neighborhood of a point x of radius r will be $V_r(x)$. A set y is *semilocally connected*, provided that for any $y \in Y$ and $\epsilon > 0$ there exists an open set U about y of diameter $< \epsilon$ such that $Y - U$ has only finitely many components. A mapping $f(X) = Y$ is *monotone*, provided that $f^{-1}(y)$ is a continuum for each $y \in Y$.

THEOREM. *Let X and Y be continua where Y is a cyclic and semilocally connected. Let $f_n(x)$ be a sequence of monotone mappings of X onto Y. If there exists a nonconstant mapping $f: X \to Y$ such that for each $x \in X$ there exists an arbitrarily small open set U about x with boundary C such that*

$$\limsup [f_n(C)] \subset f(C),$$

then $f_n(x)$ converges uniformly to $f(x)$ on X and, in case Y is locally connected, f is monotone.

Proof: We show first that $f_n(x)$ converges uniformly to $f(x)$ on X. Suppose this is not so. Then for some $\epsilon > 0$, which we may suppose chosen so that $6\epsilon < \delta[f(X)]$,

and each n there exists an $x_n \in X$ and an integer $k_n \geq n$ such that, if $z_n = f_{k_n}(x_n)$ and $y_n = f(x_n)$, we have

$$\rho(y_n, z_n) > 2\epsilon. \tag{1}$$

By taking a subsequence if necessary, we may suppose $x_n \to x \in X$, and from this we have $y_n \to y = f(x)$ by continuity of f.

By semilocal connectedness of Y together with the fact that y is not a cut point of Y, there exists a continuum Q in $Y - y$ which contains $Y - V_\epsilon(y)$. Let $\sigma < \epsilon$ be chosen so that $Q \cdot V_\sigma(y) = 0$.

Now let U be an open set containing x such that

$$f(\bar{U}) \subset V\sigma(y), \tag{2}$$

and such that lim sup $[f_n(C)] \subset f(C)$, where C is the boundary of U. Then there exists an integer N_1 such that, for any $n > N_1$,

$$f_n(C) \subset V_\sigma(y). \tag{3}$$

We show next that there exists an integer N_2 such that, for any $n > N_2$,

$$f_n(X - \bar{U}) \cdot Q \neq 0. \tag{4}$$

To this end, let z be a point of X with $f(z) \in Y - Y_{2\epsilon}(y)$ and let V be a neighborhood of z small enough that $f(\bar{V}) \subset Y - V_{2\epsilon}(y)$ and so that, if J is the boundary of V,

$$\text{lim sup } [f_n(J)] \subset f(J).$$

Then there exists an integer N_2 such that, for any $n > N_2$,

$$f_n(J) \subset Y - V_\epsilon(y) \subset Q,$$

and, since $J \subset X - \bar{U}$, by equation (2), this gives formula (4).

Now let $N = N_1 + N_2$. Then, for any $n > N$, we must have

$$f_n(U) \subset V_\epsilon(y); \tag{5}$$

for otherwise we would have $f_n(U) \cdot Q \neq 0$ so that, by formula (4),

$$f_n^{-1}(Q) \cdot U \neq 0 \neq f_n^{-1}(Q) \cdot (X - \bar{U}).$$

Since $f_n^{-1}(Q)$ is connected, this gives $f_n^{-1}(Q) \cdot C \neq 0$, so that $f_n(C) \cdot Q \neq 0$, contrary to formula (3).

Now for all n sufficiently large, say $n > N_3 > N$, we have $x_n \in U$ and $y_n \in V_\epsilon(y)$. However, this gives $z_n = f_{k_n}(x_n) \subset f_n(U) \subset V_\epsilon(y)$, so that $\rho(y_n, z_n) < 2\epsilon$, contrary to formula (1). Thus the supposition of nonuniform convergence of $f_n(x)$ to $f(x)$ leads to a contradiction. That $f(x)$ is monotone in case Y is locally connected now follows by a known theorem.[1]

3. *Regular Curve Domain Space.*—In case the domain space X of the mappings in our sequence is a regular curve in the Menger-Urysohn sense,[2] the theorem just proved has special interest because the main condition in the theorem is then a consequence of simple pointwise convergence of the sequence. For in this case $x \in X$ is contained in an arbitrarily small neighborhood U in X whose boundary C consists of just a finite number of points. Thus, if the sequence $f_n(z)$ converges at each $z \in C$ to $f(z)$, it follows that

$$\lim_{n \to \infty} f_n(C) = f(C),$$

since C contains only a finite number of points. By this reasoning we see, however, that pointwise convergence of the sequence is not required at *all points* $x \in X$. It suffices to have pointwise convergence at each point of a *regularity basis* for X. A subset X_0 of a regular curve X is a *regularity basis* for X, provided that, if $x \in X$ and ϵ is any positive number, there exists an open set U in X containing x and of diameter $< \epsilon$ whose boundary consists of just a finite number of points, each of which belongs to X_0. Thus we have the

THEOREM. *Let X be a regular curve, let Y be cyclic and suppose $f_n(x)$ is a sequence of monotone mappings of X onto Y. If there exists a nonconstant mapping $f: X \to Y$ such that $f_n(x)$ converges to $f(x)$ at each point of a regularity basis for X, then $f_n(x)$ converges uniformly to $f(x)$ on X, and $f(x)$ is monotone and onto.*

In this situation Y is necessarily locally connected; in fact, it is also a regular curve, since it is the monotone image of X by hypothesis. That f maps X *onto* Y follows from the uniform convergence, of course.

Now in any regular curve there exist *countable* regularity bases. Indeed, by a known theorem[3] any subset X_0 of a regular curve X is a regularity basis for X, provided that it contains all local separating points of X of order > 2 (a countable set) and is dense on every free arc in X. Thus, in particular, if X is a circle, *any subset* of X which is dense in X is a regularity basis for X so that we have the

COROLLARY. *If X is a circle and the sequence $f_n(x)$ of monotone mappings of X onto Y converges at each point of an everywhere dense set in X to a nonconstant mapping $f: X \to Y$, then $f_n(x)$ converges uniformly to $f(x)$ on X.*

It is unnecessary to assume Y cyclic here because, as the monotone image of X, Y is necessarily either a single point or a simple closed curve. The former case is excluded by the nonconstancy of f; and in the latter case Y is cyclic and the theorem applies.

It will be noted that this corollary includes as a very special case the result concerning sequences of homeomorphisms stated by way of illustration above in Section 1.

4. *The Noncyclic Compact Case.*—Removal of the condition that the range space Y be cyclic introduces only minor difficulties, which are easily handled. This leads to the following theorem, which, as will be indicated, can be applied to sequences of real functions. The theorem stated above cannot be so applied, since in this case the range space is a real interval.

THEOREM. *If X and Y are continua and Y is semilocally connected, then any sequence $[f_n(x)]$ of monotone mappings of X onto Y such that there exists a mapping $f: X \to Y$ satisfying:*

(1) *for each $y \in Y$, $f(X)$ intersects each component of $Y - y$, and*
(2) *for each $x \in X$ and $\epsilon > 0$ there exists an ϵ-neighborhood U of x with boundary C such that lim sup $[f_n(C)] \subset f(C)$,*

converges uniformly to $f(x)$ on X and, in case Y is locally connected, f, is monotone.

NOTES. *a*) If Y is cyclic and f is nonconstant, (1) is satisfied.
b) If f is onto, (1) is satisfied.

c) If X is a regular curve and $f_n(x) \to f(x)$ for all x on a regularity basis for X, then (2) is satisfied.

d) *Example.* There exists a sequence of homeomorphisms of the interval (0,1) onto itself which converges to the homeomorphism $y = x/2$ everywhere on (0,1) except at the right-hand end. This is readily accomplished by defining $h_n(x)$ for each $n > 0$ as follows:

$$h_n(x) = x/2 \text{ for } 0 \leq x \leq n - 1/n.$$
$$h_n(1) = 1.$$
$$h_n(x) \text{ linear for } n - 1/n < x < 1.$$

Here (2) is satisfied but (1) is not. The convergence fails to be uniform and f is not onto.

e) If X and Y are real intervals, f is onto and $f_n \to f$ on an everywhere dense set in X, both (1) and (2) are satisfied.

f) If X and Y are real intervals and $f_n \to f$ on an everywhere dense set in X, including both end points of X, the conclusions of the theorem hold.

5. *The Noncompact Case.*—A real-valued continuous function on an interval (a,b) generates a monotone mapping of (a,b) into the Y-axis if and only if it is either monotone nondecreasing or monotone nonincreasing. Thus, by redefining the functions near the ends of the interval when necessary, the following theorem concerning sequences of real functions is readily obtainable from the theorem and note f stated in Section 4.

THEOREM. *Let the sequence of real continuous and monotone nondecreasing functions $f_n(x)$ on the interval (a,b) converge on an everywhere dense set including a and b to a function $f(x)$, where $f(x)$ is continuous on (a,b). Then $f_n(x)$ converges uniformly to $f(x)$ on (a,b) and $f(x)$ is monotone.*

Of course, this theorem can be proved directly by standard techniques and with little difficulty. It is possible, however, to extend the theorem proved in Section 2 to a much broader setting, requiring neither the compactness of the domain space nor the cyclic property of the range space and, further, permitting the image sets under the mappings in the sequence to be different. This result applies directly to give the above theorem on real functions and, at the same time, is general enough to permit possible application to sequences of functions of a complex variable defined and continuous in a region.

DEFINITION. If A_n is a sequence of sets in a metric space, we define

$$\limsup A_n \subset_s A,$$

read "limit superior of A_n strictly contained in A," to mean that, for any $\epsilon > 0$, almost all the sets A_n lie entirely in $V_\epsilon(A)$. In a compact space this is clearly equivalent to the, in general, weaker statement "$\limsup A_n \subset A$."

THEOREM. *Let the closed generalized continua X, Y, and Y' in a locally compact separable metric space be such that $Y' \subset Y$ and for each $y \in Y$, Y' intersects each component of $Y - y$. Let the sequence of compact monotone mappings $f_n(X) = Y_n$ and the mapping $f(X) = Y'$ satisfy*

a) the sets Y_n converge 0-regularly to Y and

b) for each $x \in X$ and $\epsilon > 0$ there exists an ϵ-neighborhood U of x with boundary C such that lim sup $f_n(C) \subset_s f(C)$.

Then $[f_n(x)]$ converges almost uniformly to $f(x)$ on X and if each $f^{-1}(y)$, $y \in Y'$, has a nonempty compact component, f is compact and monotone.

A *generalized continuum* is a locally compact connected set. A mapping $g:A \to B$ is *compact* provided that $g^{-1}(K)$ is compact for every compact set $K \subset B$. Condition (a) in this theorem, 0-regular convergence of Y_n to Y, means that Y_n converges to Y and for any $\epsilon > 0$ a $\delta > 0$ and an integer N exist such that if $n > N$ any pair of points $x,y \in Y_n$ with $\rho(x,y) < \delta$ lie together in a continuum in Y_n of diameter $< \epsilon$. This condition is automatically satisfied in case all the Y_n are the same, and thus the same as Y, provided that Y is uniformly locally connected. However, this condition, as well as all others in the theorem, is also satisfied in the situation covered by the theorem on sequences of real functions stated at the beginning of this section. For in this case if $c = f(a)$, $d = f(b)$, $c_n = f_n(a)$, $d_n = f_n(b)$, Y_n is the interval (c_n,d_n), and the intervals (c_n,d_n) converge 0-regularly to the interval (c,d). Finally, *almost uniform convergence* as usual means convergence which is uniform on each compact subset of X.

The proof for this theorem is somewhat more complicated than that given in Section 2. Hence it is not given here but will appear in the later treatment mentioned above.

6. *Quasi-open Mappings.*—For sequences of some types of mappings other than monotone ones, similar results are obtainable. This is true, in particular, of (strongly) quasi-open mappings, i.e., mappings $f:X \to Y$ such that any $y \in Y$ is interior to $f(U)$, where U is any open set in X which contains a nonempty compact component of $f^{-1}(y)$. Such mappings are significant in connection with functions of a complex variable, since not only all analytic functions but also all continuous functions satisfying simple minimum modulus conditions generate quasi-open mappings. Results will be only sketched here.

Let X and Y be locally connected generalized continua and suppose Y is cyclic and noncompact. (More generally, Y could be taken such that for each $y \in Y$, each component of $Y - y$ is noncompact.) Consider the following conditions:

$$(*) \qquad Fr[f(R)] \subset f[Fr(R)]$$

for mappings $f:X \to Y$ and regions (i.e., connected open sets) R in X, where Fr denotes the boundary; and

$$(\dagger) \qquad \lim_{n \to \infty} [f_n(C)] = [\lim_{n \to \infty} f_n](C).$$

for sequences of mappings $f_n:X \to Y$ and subsets C of X. Condition (*) is a commutative condition and (†) is an associativity condition. We note first that *a mapping $f:X \to Y$ is (strongly) quasi-open if and only if* it satisfies (*) on every conditionally compact region R in X.

To have meaning, condition (†) would require that the sets $[f_n(C)]$ actually converge to a set A and that the mappings f_n converge to a limit mapping f and that

$A = f(C)$. For our purposes we replace condition (†) by the less restricting but closely related condition

(††) $\qquad \limsup [f_n(C)] \subset_s f(C),$

where $f:X \to Y$ is a mapping related in some specified way to the sequence of mappings $f_n:X \to Y$. We may then state the

THEOREM. *Let $f_n:X \to Y$ be a sequence of quasi-open mappings and suppose there exists a mapping $f:X \to Y$ with point inverses having compact components such that for each $x \in X$ there exists an arbitrarily close region R in X with boundary C about the component of $f^{-1}f(x)$ containing x such that (††) holds on C. Then $f_n(x)$ converges almost uniformly to $f(x)$ on X.*

As already indicated, this theorem has implications for sequences of functions similar to those mentioned in the earlier sections for the corresponding theorems on sequences of monotone mappings. The application of the theorem tc the case of a closed algebra of mappings of X into Y is of special interest and will receive attention in a later paper.

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[1] G. T. Whyburn, "*Regular convergence and monotone transformations*," *Am. J. Math.*, **57**, 902–906, 1935.

[2] K. Menger, *Kurventheorie* (Berlin: B. G. Teubner, 1932).

[3] L. Whyburn, Ergeb. Math. Kolloq. 2, p. 11, 1930. This theorem may be found also in Menger's *Kurventheorie*, pp. 278–279.

SOLUTION OF THE BOLTZMANN-HILBERT INTEGRAL EQUATION II. THE COEFFICIENTS OF VISCOSITY AND HEAT CONDUCTION

BY CHAIM L. PEKERIS AND ZIPORA ALTERMAN

DEPARTMENT OF APPLIED MATHEMATICS, WEIZMANN INSTITUTE, REHOVOT, ISRAEL

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1. *Introduction.*—In this investigation we apply the method developed in Part I[1] to the determination of the coefficients of viscosity and heat conduction and of the perturbations in the respective distribution functions in a simple gas of the rigid sphere model. The essence of the method consists in *reducing the Boltzmann-Hilbert integral equation*, which controls the distribution function $f(\mathbf{c}, \mathbf{r}, t)$ of the molecules in space $\mathbf{r}$ and in velocity-space $\mathbf{c}$, *to an ordinary differential equation.* In the cases of viscosity and heat conduction, the respective differential equations are of the fourth order. These are integrated numerically on the electronic computer (WEIZAC) to obtain the distribution functions, and from the latter the coefficients of viscosity and heat conduction are evaluated.

Let the gas molecules be rigid spheres of diameter σ and mass m. Further, let

$$f^0 = n\left(\frac{m}{2\pi kT}\right)^{3/2} \exp\left(-\frac{mC^2}{2kT}\right), \qquad \mathbf{C} = \mathbf{c} - \mathbf{c}_0,$$

$$f = f^0(1 + \varphi), \qquad \boldsymbol{p} = \boldsymbol{C}\sqrt{\frac{m}{2kT}}, \tag{1}$$

$$\mathfrak{D}f = \frac{\partial f}{\partial t} + \boldsymbol{c}\cdot\frac{\partial f}{\partial \boldsymbol{r}} + \boldsymbol{F}\cdot\frac{\partial f}{\partial \boldsymbol{c}}, \tag{2}$$

where T denotes the temperature of the gas, n the number density, k Boltzmann's constant, and $\boldsymbol{F}$ the force per unit mass acting on the molecules. Then on treating φ as a small quantity of the first order, the Boltzmann-Hilbert integral equation takes on the form

$$\mathfrak{D}f^0 = -\frac{\sigma^2 n^2 m}{2\pi kT}\left\{M(p)e^{-2p^2}\varphi(\boldsymbol{p}) + \frac{1}{\pi e}e^{-p^2}\int \varphi(\boldsymbol{p}_1)e^{-p_1^2}\left(R - \frac{2}{R}e^{\omega^2}\right)d\boldsymbol{p}_1\right\} \tag{3}$$

where

$$M(p) = 1 + \left(2p + \frac{1}{p}\right)P(p), \qquad P(p) = e^{p^2}\int_0^p e^{-x^2}dx, \tag{4}$$

$$R = |\boldsymbol{p} - \boldsymbol{p}_1|, \qquad \omega = \frac{pp_1\sin\theta'}{R}, \tag{5}$$

and θ' denotes the angle between $\boldsymbol{p}$ and $\boldsymbol{p}_1$.

In evaluating $\mathfrak{D}f^0$, we allow for variation in space of the quantities n, $\boldsymbol{C}$, and T, obtaining

$$\mathfrak{D}f^0 = f^0\left\{\frac{1}{n}\boldsymbol{C}\cdot\frac{\partial n}{\partial \boldsymbol{r}} + \left(p^2 - \frac{5}{2}\right)\boldsymbol{C}\cdot\frac{\partial \ln T}{\partial \boldsymbol{r}} + 2\left(p_i p_k - \frac{1}{3}p^2\delta_{ik}\right)\frac{\partial u_{0i}}{\partial x_k}\right\}. \tag{6}$$

Here p_i denotes the components of $\boldsymbol{p}$, and u_{0i} the components of $\boldsymbol{c}_0$. The first term in equation (6) is proportional to the density gradient and enters into the determination of the coefficient of self-diffusion, which was treated in Part I. In this investigation we shall treat the problems of heat conduction and viscosity, for which $\mathfrak{D}f^0$ is represented by the second and third terms, respectively, in equation (6).

2. *The Differential Equation for the Distribution Function in the Case of Viscosity.*—In treating viscosity, we let n and T be constant, so that $\mathfrak{D}f^0$ reduces to the terms proportional to the velocity gradients in equation (6). An appropriate form for φ in this case is

$$\varphi = -\frac{1}{n\sigma^2}\sqrt{\frac{2m}{\pi kT}}\,b(p)\left(p_i p_k - \frac{1}{3}p^2\delta_{ik}\right)\frac{\partial u_{0i}}{\partial x_k}, \tag{7}$$

where the summation convention is adhered to. On substituting equations (6) and (7) in equation (3) and equating the coefficients of $\partial u_{0i}/\partial x_k$, we get

$$\begin{aligned} p_i p_k - \frac{1}{3}p^2\delta_{ik} = \Bigg[&\left(p_i p_k - \frac{1}{3}p^2\delta_{ik}\right)M(p)e^{-p^2}b(p) \\ &+ \frac{1}{\pi}\int\left(p_n p_{k1} - \frac{1}{3}p_1^2\delta_{ik}\right)b(p_1)e^{-p_1^2}\left(R - \frac{2}{R}e^{\omega^2}\right)d\boldsymbol{p}_1\Bigg]. \end{aligned} \tag{8}$$

It is convenient to let $i = k = 3$ in equation (8), whereby it takes on the form

$$p^2P_2(\cos\theta) = \left[p^2M(p)e^{-p^2}b(p)P_2(\cos\theta) + \frac{1}{\pi}\int p_1^2P_2(\cos\theta_1)b(p_1)e^{-p_1^2}\left(R - \frac{2}{R}e^{\omega^2}\right)d\mathbf{p}_1\right]. \tag{9}$$

As was pointed out in Part I, this integral equation involves only the component $A_2(p, p_1)$ of the expansion of the kernel $[R - (2/R)e^{\omega^2}]$ into spherical harmonics of the angle θ' between $\mathbf{p}$ and $\mathbf{p}_1$:

$$\left[R - \left(\frac{2}{R}\right)e^{\omega^2}\right] = \sum_{n=0}^{\infty}\left(n + \frac{1}{2}\right)A_n(p, p_1)P_n(\cos\theta'). \tag{10}$$

Using equation (10) in equation (9), we obtain

$$\int p_1^2e^{-p_1^2}b(p_1)\left(R - \frac{2}{R}e^{\omega^2}\right)P_2(\cos\theta_1)d\mathbf{p}_1 = 2\pi P_2(\cos\theta)\int_0^{\infty} p_1^4e^{-p_1^2}b(p_1)A_2(p, p_1)d\mathbf{p}_1, \tag{11}$$

whereby the three-dimensional integral equation (9) reduces to the following one-dimensional integral equation:

$$p^2 = p^2M(p)e^{-p^2}b(p) + 2\int_0^{\infty} e^{-p_1^2}p_1^4A_2(p, p_1)b(p_1)dp_1. \tag{12}$$

The kernel $A_2(p, p_1)$, which is symmetrical in the arguments p and p_1, was previously[2] evaluated in the explicit form

$$p^3p_1^3A_2(p, p_1) = \left[\frac{2}{35}p_1^7 - 3p_1^3 + 18p_1 + (-6p_1^4 + 15p_1^2 - 18)P(p_1)\right] + p^2\left[-\frac{2}{15}p_1^5 + 3p_1 + (2p_1^2 - 3)P(p_1)\right], \qquad p_1 < p, \tag{13}$$

$$= \left[\frac{2}{35}p^7 - 3p^3 + 18p + (-6p^4 + 15p^2 - 18)P(p)\right] + p_1^2\left[-\frac{2}{15}p^5 + 3p + (2p^2 - 3)P(p)\right], \qquad p_1 > p. \tag{14}$$

Putting

$$\nu(p) \equiv p^5 - p^5M(p)e^{-p^2}b(p), \tag{15}$$

the integral equation (12) takes on the form

$$\nu(p) = \int_0^p e^{-x^2}b(x)\left]\frac{4}{35}x^8 - 6x^4 + 36x^2 + (-12x^5 + 30x^3 - 36x)P(x)\right]dx + p^2\int_0^p e^{-x^2}b(x)\left[-\frac{4}{15}x^6 + 6x^2 + (4x^3 - 6x)P(x)\right]dx$$

$$+\left[-\frac{4}{15}p^5+6p+(4p^2-6)P(p)\right]\int_p^\infty e^{-x^2}b(x)x^3dx \tag{16}$$

$$+\left[\frac{4}{35}p^7-6p^3+36p+(-12p^4+30p^2-36)P(p)\right]\int_p^\infty e^{-x^2}b(x)xdx.$$

We now proceed to reduce this integral equation to a differential equation. We have

$$\frac{1}{p}\frac{dv}{dp}=\int_0^p e^{-x^2}b(x)\left[-\frac{8}{15}x^6+12x^2+(8x^3-12x)P(x)\right]dx$$

$$+\left[-\frac{4}{3}p^3+4p+(8p^2-4)P(p)\right]\int_p^\infty e^{-x^2}x^3b(x)dx$$

$$+\left[\frac{4}{5}p^5-12p^3+12p+(-24p^4+12p^2-12)P(p)\right]\int_p^\infty e^{-x^2}b(x)xdx. \tag{17}$$

Let

$$y(p)=-\frac{1}{2}p^2\int_p^\infty e^{-x^2}b(x)xdx+\frac{1}{2}\int_p^\infty e^{-x^2}b(x)x^3dx, \tag{18}$$

so that

$$\int_p^\infty e^{-x^2}b(x)xdx=-\frac{y}{p}, \tag{19}$$

$$\int_p^\infty e^{-x^2}b(x)x^3dx=2y-p\dot{y},\qquad e^{-p^2}p^3b(p)=p\ddot{y}-\dot{y}, \tag{20}$$

the dots denoting differentiation with respect to p.

Using these substitutions in equation (17), we are left with only the first integral, and the latter can be reduced by an additional differentiation, yielding the following differential equation for the determination of $y(p)$:

$$[p^2+(p+2p^3)P(p)]y^{IV}+[6p+4p^3+(2+16p^2+8p^4)P(p)]\dddot{y}$$

$$+\left[1+20p^2+4p^4+\left(-\frac{1}{p}+10p+44p^3+8p^5\right)P(p)\right]\ddot{y}$$

$$+\left[-\frac{1}{p}+4p+12p^3+\left(\frac{1}{p^2}-10+20p^2+24p^4\right)P(p)\right]\dot{y} \tag{21}$$

$$+[8p^2+(16p+32p^3)P(p)]y=15p^2.$$

We are interested in a solution of equation (21) which is regular in the whole range of p from 0 to ∞.

A result similar to, but not identical with, equation (21) was obtained by Boltzmann[3] toward the end of his third paper on viscosity. Boltzmann derived an equation[4] which is equivalent to equation (17) above; however, in deducing from it the fourth-order differential equation for y, he committed several errors. His equation (38) should read

$$15x = [2x^3 + (x^2 + 2x^3)\xi]Y^{IV} + [12x^2 + 4x^3 + (4x + 14x^2 + 4x^3)\xi]\dddot{Y} + [11x + 16x^2 + 2x^3 + (2 + 16x + 17x^2 + 2x^3)\xi]\ddot{Y} + [6x + 4x^2 + (8x + 4x^2)\xi]\dot{Y} + [x + (1 + 2x)\xi]Y, \quad (22)$$

where

$$x = p^2, \qquad \xi = 2pP(p), \qquad Y(x) = 8y(p). \quad (23)$$

Equation (22) is identical with equation (21).

The method by which Boltzmann arrived at the differential equation (22) is extremely laborious, the derivation covering 160 pages, of which over 40 are pure mathematics,[5] in the sense that they are either entirely word-pure or are adorned with just one sentence.

3. *The Differential Equation for the Distribution Function in the Case of Heat Conduction.*—With n and $\mathbf{c}_0$ constant in space, equation (6) reduces to

$$\mathfrak{D}f^0 = f^0\left(p^2 - \frac{5}{2}\right)\mathbf{C}\cdot\frac{\partial \ln T}{\partial \mathbf{r}} = \sqrt{\frac{2kT}{m}}\, f^0\left(p^2 - \frac{5}{2}\right)\mathbf{p}\cdot\frac{\partial \ln T}{\partial \mathbf{r}}. \quad (24)$$

A suitable form for φ now is

$$\varphi = -\frac{1}{n\sigma^2\sqrt{\pi}}\, a(p)\mathbf{p}\cdot\frac{\partial \ln T}{\partial \mathbf{r}}. \quad (25)$$

When equations (24) and (25) are substituted in equation (3) and the coefficients of $\partial T/\partial \mathbf{r}$ are equated, we get the following integral equation for the determination of $a(p)$:

$$M(p)e^{-p^2}a(p)\mathbf{p} + \frac{1}{\pi}\int a(p_1)\mathbf{p}_1 e^{-p_1^2}\left(R - \frac{2}{R}e^{\omega^2}\right)d\mathbf{p}_1 = \left(p^2 - \frac{5}{2}\right)\mathbf{p}. \quad (26)$$

By now taking $\mathbf{p}$ in the direction of z, equation (26) assumes the form

$$pM(p)e^{-p^2}a(p)P_1(\cos\theta) + \frac{1}{\pi}\int p_1 a(p_1)e^{-p_1^2}P_1(\cos\theta_1)\left(R - \frac{2}{R}e^{\omega^2}\right)d\mathbf{p}_1 = \left(p^3 - \frac{5}{2}p\right)P_1(\cos\theta). \quad (27)$$

This equation evidently involves only the first harmonic $A_1(p, p_1)$ in the expansion (10) of the kernel $[R - (2/R)e^{\omega^2}]$:

$$\int p_1 a(p_1)e^{-p_1^2}P_1(\cos\theta_1)\left(R - \frac{2}{R}e^{\omega^2}\right)d\mathbf{p}_1 = 2\pi P_1(\cos\theta)\int_0^\infty p_1^3 a(p_1)e^{-p_1^2}A_1(p, p_1)dp_1, \quad (28)$$

so that equation (27) reduces to

$$pM(p)e^{-p^2}a(p) + 2\int_0^\infty e^{-p_1^2}p_1^3 a(p_1)A_1(p, p_1)dp_1 = p^3 - \frac{5}{2}p. \quad (29)$$

Here[2]

$$p^2p_1^2A_1(p, p_1) = \frac{2}{15}\,p_1^5 - 4g(p_1) - \frac{2}{3}\,p_1^3p^2, \qquad p_1 < p, \tag{30}$$

$$= \frac{2}{15}\,p^5 - 4g(p) - \frac{2}{3}\,p^3p_2^1, \qquad p_1 > p, \tag{31}$$

where

$$g(p) = [p + (p^2 - 1)P(p)], \qquad P(p) = e^{p^2}\int_0^p e^{-x^2}\,dx. \tag{32}$$

Thus $a(p)$ is a solution of the one-dimensional integral equation

$$\gamma(p) \equiv p^5 - \tfrac{5}{2}p^3 - p^3M(p)e^{-p^2}a(p)$$

$$= \int_0^p e^{-x^2}a(x)x\left[\frac{4}{15}\,x^5 - 8g(x) - \frac{4}{3}\,p^2x^3\right]dx$$

$$+ \int_p^\infty e^{-x^2}a(x)x\left[\frac{4}{15}\,p^5 - 8g(p) - \frac{4}{3}\,p^3x^2\right]dx. \tag{33}$$

As in the previous section, we proceed to turn the integral equation (33) into a differential equation by first introducing an auxiliary function,

$$S(p) = -\int_p^\infty e^{-x^2}\,xa(x)dx. \tag{34}$$

Repeated differentiation of equation (33) yields

$$\frac{d^2}{dp^2}\left(\frac{1}{p}\frac{d\gamma}{dp}\right) = 8\,\frac{d^2}{dp^2}\left(\frac{S}{p}\frac{dg}{dp}\right) - 8pS, \tag{35}$$

or

$$[p^2 + (2p^3 + p)P(p)]S^{IV} + [7p + 6p^3 + (1 + 20p^2 + 12p^4)P(p)]\dddot{S}$$

$$+ \left[2 + 42p^2 + 12p^4 + \left(-\frac{2}{p} + 26p + 96p^3 + 24p^5\right)P(p)\right]\ddot{S} \tag{36}$$

$$+ \left[-\frac{2}{p} + 38p + 72p^3 + 8p^5 + \left(\frac{2}{p^2} - 2 + 140p^2 + 152p^4 + 16p^6\right)P(p)\right]\dot{S}$$

$$+ [56p^2 + 32p^4 + (32p + 160p^3 + 64p^5)P(p)]S = 30p^2.$$

The solution $S_1(p)$ of equation (36), which is required for our application, is to be regular in the whole range of p extending from 0 to ∞. In addition to $S_1(p)$, the homogeneous differential equation (36) also possesses the regular solution

$$S_0(p) = Ae^{-p^2}, \tag{37}$$

so that

$$S(p) = S_1(p) + Ae^{-p^2}. \tag{38}$$

The availability of $S_0(p)$ enables us to satisfy a condition on $S(p)$:

$$\int_0^\infty S(p)p^2dp = 0, \tag{39}$$

which is required[6] in order that

$$\int f^0 m\mathbf{C}d\mathbf{c} = \int fm\mathbf{C}d\mathbf{c}. \tag{40}$$

Equation (40) expresses the normalization condition that the mean flow be given by $\mathbf{c}_0$, even in the presence of the temperature gradient.

4. *The Coefficients of Heat Conduction and Viscosity.*—The flow of heat, $\boldsymbol{q}$, is given by

$$\begin{aligned}\boldsymbol{q} = \frac{m}{2}\int f^0\varphi C^2\mathbf{C}d\mathbf{c} &= -\frac{m}{2\pi^2\sigma^2}\left(\frac{2kT}{m}\right)^{5/2}\int e^{-p^2}p^2\left(\mathbf{p}\cdot\frac{\partial \ln T}{\partial \mathbf{r}}\right)\mathbf{p}a(p)d\mathbf{p} \\ &= -\frac{4k}{3\pi\sigma^2}\sqrt{\frac{2kT}{m}}\frac{\partial T}{\partial \mathbf{r}}\int_0^\infty e^{-p^2}a(p)p^6dp = -\lambda\frac{\partial T}{\partial \mathbf{r}}.\end{aligned} \tag{41}$$

Hence we get for the coefficient of heat conduction, λ,

$$\lambda = \frac{4k}{3\pi\sigma^2}\sqrt{\frac{2kT}{m}}\int_0^\infty e^{-p^2}a(p)p^6dp. \tag{42}$$

In the case of viscosity, the non-hydrostatic part of the stress tensor, τ^1_{jl} is expressed by

$$\begin{aligned}\tau^1_{jl} = m\int f^0\varphi C_jC_l d\mathbf{c} &= \\ -\frac{2kT}{\pi^2\sigma^2}\sqrt{\frac{2m}{kT}}\int e^{-p^2}b(p)&\left(p_ip_k - \frac{1}{3}p^2\delta_{ik}\right)\frac{\partial u_{0i}}{\partial x_k}p_jp_l d\mathbf{p}.\end{aligned} \tag{43}$$

When this is evaluated, we obtain expressions for $\tau_{jl}{}^1$ of the Stokes-Navier form, with a coefficient of viscosity, μ, given by

$$\mu = \frac{8\sqrt{2mkT}}{15\pi\sigma^2}\int_0^\infty e^{-p^2}b(p)p^6dp. \tag{44}$$

5. *Integration of the Differential Equations.*—Taking first the differential equation (36) for the distribution function $S(p)$ in the case of heat conduction, we find from the indicial equation at the origin the following allowed values for the leading powers n of p:

$$n = -1, 0, 1, 2. \tag{45}$$

Of these, the value 1 is disallowed, because, according to equation (34), $\dot{S}(0)$ must vanish; and so is also the value $n = -1$. The index $n = 0$ gives the solution (37), and there is another regular solution, $S_2(p)$, starting with p^2. The inhomogeneous solution $S_4(p)$ of equation (36) starts with the power p^4.

At infinity, there are, in addition to equation (37), four solutions with the following leading terms in their asymptotic expansions:

$$pe^{-p^2}, \quad \frac{1}{p}e^{-p^2}, \quad \frac{1}{p^2}e^{-p^2}, \quad \frac{1}{p^4}, \tag{46}$$

of which the first is the inhomogeneous solution. Both $S_2(p)$ and $S_4(p)$ were found upon numerical integration to approach the $(1/p^4)$-solution for large values of p. The ratio of $S_2(p)$ to $S_4(p)$ therefore approaches a constant, whereby one can determine a linear combination of $S_2(p)$ and $S_4(p)$ which is free from the $(1/p^4)$-solution. This procedure was found to be sufficiently accurate to obviate the need of integrating the first three solutions in (46) backward from large values of p, and then matching them at some intermediate value with a linear combination of $S_2(p)$ and $S_4(p)$.

The solution $S(p)$ thus obtained, regular in the whole range of p, is shown in Table 1. Expression (42) for the coefficient of heat conduction, λ, can be written in the non-dimensional form,

$$\frac{\lambda}{\lambda_1} = -\frac{256}{45}\sqrt{\frac{2}{\pi}}\int_0^\infty p^4 S(p)dp, \tag{47}$$

where

$$\lambda_1 = \frac{75k}{64\sigma^2}\sqrt{\frac{kT}{\pi m}}. \tag{48}$$

Using the values of $S(p)$ obtained by the numerical integration of equation (36), we obtain a value of 1.025218 for (λ/λ_1). The values for (λ/λ_1) obtained in the successive approximations of the Chapman-Enskog method[7] are

$$1, \quad 1.02273, \quad 1.02482, \quad 1.02513. \tag{49}$$

In the case of the distribution function $y(p)$ for viscosity, which is governed by the differential equation (21), we find, from the indicial equation at the origin, the following allowed values for the leading powers n of p:

$$n = -1, 0, 1, 2, \tag{50}$$

of which, in addition to $n = -1$, the value $n = 1$ is also disallowed on account of the requirement, resulting from equation (19), that $\dot{y}(0)$ must vanish. Near the origin we need to integrate the solutions $y_0(p)$ and $y_2(p)$, belonging to $n = 0$ and $n = 2$, respectively, as well as the inhomogeneous solution $y_4(p)$ starting with p^4.

For large values of p, the leading terms in the asymptotic solutions of equation (21) are

$$p^{-1}e^{-p^2}, \quad p^{-3-i\sqrt{3}}e^{-p^2}, \quad p^{-3+i\sqrt{3}}e^{-p^2}, \quad p^{-1-i\sqrt{3}}, \quad p^{-1+i\sqrt{3}}, \tag{51}$$

of which the first is the inhomogeneous solution. Asymptotically, the solutions $y_0(p)$, $y_2(p)$, and $y_4(p)$ are dominated by the last two solutions of (51). As in the case of heat conduction, these solutions were eliminated by using the limiting values of the cross-ratios of $y_0(p)$, $y_2(p)$, and $y_4(p)$ for large p. The resulting solution, $y(p)$, thus obtained, regular in the whole range of p, is shown in Table 1.

The expression for the coefficient of viscosity μ given in equation (44) can be put in the form

$$\frac{\mu}{\mu_1} = \frac{256}{5\sqrt{2\pi}}\int_0^\infty p^2 y(p)dp, \tag{52}$$

with

$$\mu_1 = \frac{5\sqrt{kmT}}{16\sqrt{\pi}\sigma^2}. \tag{53}$$

Values for (μ/μ_1) obtained in the successive approximations of the Chapman-Enskog method[7] are

$$1, \quad 1.01485, \quad 1.01588, \quad 1.01600. \tag{54}$$

Using the values of $y(p)$ obtained by numerical integration of equation (21), we obtain the value of 1.016034 for (μ/μ_1).

In the first-order theory presented in this paper it was assumed that φ in equation (1) is less than 1. We see from Table 1 that the quantities $p^2b(p)$ and $pa(p)$, which

TABLE 1*

$$p = C\sqrt{\frac{m}{k2T}}$$

p	$S(p)$	$pa(p)$	$y(p)$	$p^2b(p)$
0	0.597142	0	0.125867	0
0.1	.586378	−0.215765	.124491	0.006196
0.2	.555074	−0.424299	.120458	0.024668
0.3	.506062	−0.618610	.114028	0.055080
0.4	.443610	−0.792152	.105611	0.096902
0.5	.372844	−0.938986	.095711	0.149447
0.6	.299102	−1.053877	.084882	0.211920
0.7	.227314	−1.132324	.073677	0.283462
0.8	.161529	−1.170543	.062599	0.363191
0.9	.104613	−1.165432	.052070	0.450239
1.0	.058160	−1.114455	.042407	0.543772
1.1	.022569	−1.015593	.033820	0.643015
1.2	−.002738	−0.867241	.026416	0.747251
1.3	−.019035	−0.668134	.020210	0.855835
1.4	−.027987	−0.417277	.015146	0.968190
1.5	−.031383	−0.113887	.011121	1.083802
1.6	−.030923	0.242651	.008000	1.202218
1.7	−.028074	.652825	.005639	1.323043
1.8	−.024002	1.117023	.003895	1.445929
1.9	−.019556	1.635558	.002637	1.570574
2.0	−.015295	2.208681	.001749	1.696714
2.1	−.011537	2.836601	.001138	1.824122
2.2	−.008421	3.519490	.000725	1.952596
2.3	−.005962	4.257492	.000453	2.081966
2.4	−.004101	5.050729	.000277	2.212081
2.5	−.002744	5.899307	.000166	2.342811
2.6	−.001789	6.803316	.000098	2.474043
2.7	−.001136	7.762836	.000057	2.605678
2.8	−.000704	8.777936	.000032	2.737632
2.9	−.000426	9.848679	.000018	2.869832
3.0	−.000252	10.975	.000010	3.0022
3.1	−.000145	12.157	.000005	3.1347
3.2	−.000082	13.395	.000003	3.2673
3.3	−.000045	14.689	.000001	3.3999
3.4	−.000024	16.039	0.000001	3.5325
3.5	−.000013	17.444	...	3.6651
3.6	−.000007	18.906	...	3.7977
3.7	−.000003	20.423	...	3.9301
3.8	−.000002	21.997	...	4.0625
3.9	−0.000001	23.626	...	4.1947
4.0	...	25.319	...	4.3268
5.0	...	45	...	5.6

* The dependence of the perturbation in the distribution function on p is given by $pa(p)e^{-p^2}$ in the case of heat conduction and by $p^2b(p)e^{-p^2}$ in the case of viscosity.

represent the factors depending on p in equations (7) and (25), become large for large values of p. Hence the linearized approximation becomes relatively poorer for large values of momenta.

Summary.—In a previous publication[1] it was shown that it is possible to reduce the Boltzmann-Hilbert integral equation, occurring in the classical problem of transport phenonena in a rigid-sphere gas model, into a differential equation. In the case of self-diffusion treated there, this differential equation was of the second order, and its solution yielded a value for the coefficient of self-diffusion which was in good agreement with the value obtained by the variational Chapman-Enskog method. In this paper the method is applied to the problems of heat conduction and viscosity. In both cases the differential equations for the respective distribution functions are of the fourth order. The solution of these equations leads to values for the coefficients of heat conduction and of viscosity which are in good agreement with the values obtained by the Chapman-Enskog method. From the tabulated values of the distribution functions it follows that the linearized approximation becomes relatively poorer in the outer regions of momentum-space. A differential equation of the fourth order for the distribution function in the case of viscosity was derived by L. Boltzmann.[3] Boltzmann's differential equation is incorrect as it stands because of errors that crept into the last stages of his derivation. Boltzmann did not integrate the differential equation. To the authors' knowledge the differential equation governing the distribution function in the case of heat conduction, which is derived and solved in this paper, is new.

[1] C. L. Pekeris, these PROCEEDINGS, **41**, 661, 1955. This paper will be referred to as "Paper I."
[2] *Ibid.*, eq. (18).
[3] L. Boltzmann, *Collected Works*, **2** (1881), 545.
[4] *Ibid.*, eq. (37), p. 544. Here $\psi(p^2) = 2b(p)$.
[5] *Ibid.*, pp. 479–522.
[6] See S. Chapman and T. G. Cowling, *The Mathematical Theory of Non-uniform Gases* (Cambridge: At the University Press, 1952), pp. 109 and 121.
[7] Ibid., p. 169.

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*PROTEIN SYNTHESIS AND TISSUE INTEGRITY IN THE CORNEA OF THE DEVELOPING CHICK EMBRYO**

BY HEINZ HERRMANN

LABORATORY OF CHEMICAL EMBRYOLOGY, DEPARTMENT OF PEDIATRICS, UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER, COLORADO

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In the course of investigations of protein formation in the tissues of the chick embryo, two systems have been used so far in this laboratory for the comparison of tracer incorporation and protein accumulation in developing cells: isolated protein fractions of muscle tissue obtained from embryos developing *in ovo*[1] and the total protein moiety of early explanted embryos.[2, 3] The first approach offers advantages for the preparation of well-defined protein fractions. But the interpretation of the data obtained from *in vivo* systems is complicated by the changing relationships of the tracer levels in the blood, in the cell pool, and in the proteins of the embryo.

In explants a constant tracer supply eliminates, in part, such difficulties, but the small quantities of material available and the unknown preparative properties of embryonic proteins make the isolation of uniform protein fractions a trying task.

In searching for embryonic tissues which would permit a fuller quantitative analysis of protein synthesis, the cornea seemed of interest for several reasons. This tissue is not vascularized and can be expected to maintain, under *in vitro* conditions, its normal metabolism for a longer time than tissues which depend on a rapid supply of nutrients by a well-developed vascular bed. The mesodermal component of the cornea produces substantial amounts of one distinct protein species—collagen—which can be isolated for analysis at a relatively early phase of development. The scleral and corneal components of the ocular mesoderm develop characteristic biochemical differences[4] in the course of embryogenesis, and the analysis of conditions giving rise to these molecular distinctions is of interest for the understanding of embryonic differentiation.

In the course of the exploration of the incorporation of glycine-1-C^{14} into the proteins of the corneal stroma, the tracer uptake was found to be dependent to a large extent upon the presence of the epithelial component of the cornea. The dependence of tracer incorporation into one tissue component (stroma mesoderm) upon the presence of another cell type (epithelial ectoderm) is of possible importance from a biochemical and developmental point of view. Therefore, data demonstrating the difference in the tracer uptake into one of the protein fractions, consisting mainly of collagen, of the stroma in the intact cornea and in the absence of epithelium are reported in this communication.

Materials and Methods.—Four corneas from 10-day chick embryos and two corneas from freshly hatched chicks (24–29 days of total development) were placed in 10-ml. breakers containing 1 ml. of medium. In preparing one of the more complex nutrient solutions[5] as medium (complete medium), the natural glycine was omitted from the amino acid mixture and, instead, glycine-1-C^{14} was added to the medium just before the start of the experiment (1 μc. corresponding to about 20 μg. in 1 ml. of medium). The beakers with the tissue were incubated for 1–6 hours in a Dubnoff Shaker. After termination of the incubation, the corneas were washed with Ringers solution, and the extractable proteins were removed by extracting with six changes of 0.1 NaOH over a period of 36 hours at 4° C. The NaOH was washed off with distilled water, and the remaining NaOH-insoluble fraction (I.S.F.) was extracted with 1 ml. of distilled water in a pressure cooker at 15 lb. pressure, following essentially the procedure of Lowry *et al.*[6] for collagen preparation as used previously for embryonic tissues.[7] One aliquot of the extract was plated out for counting in a gas-flow counter (Nuclear, D 47) while the other aliquot was used for nitrogen determination.

To test the effect of the epithelium on the tracer incorporation into the stroma proteins, the cornea was excised, the epithelium was removed under a dissecting microscope, and the stroma alone was incubated. In the controls the epithelium was removed after incubation.

Results.—The dependence of the uptake of glycine-1-C^{14} into the insoluble fraction (I.S.F.) of the stroma upon the presence of the corneal epithelium is clearly evident from the figures presented in Table 1. If the epithelium was removed before incubation of the stroma in a medium containing glycine-1-C^{14}, the tracer incorpora-

TABLE 1

UPTAKE OF GLYCINE-1-C^{14} INTO COLLAGEN FRACTION OF CORNEAL STROMA OF DEVELOPING CHICK*

		Stroma with Epithelium	Stroma without Epithelium	
10-day chick embryo:				
Complete medium				
Incubation time	1 hr.	453 (8)	77 (6)	17%†
Incubation time	2 hr.	1403 (2)		
Incubation time	4 hr.	3182 (4)	574 (2)	18%
Incubation time	6 hr.	5680 (2)		
3–8-day hatched chick:				
Complete medium				
Incubation time	1 hr.	106 (16)	6 (12)	6%
Incubation time	4 hr.	386 (4)	15 (4)	4%
Incubation time	1 hr.			
$^1/_2$ epithelium removed		56 (4)		
$^3/_4$ epithelium removed		36 (4)		
Complete medium				
Incubation time	1 hr.			
Dinitrophenol				
Final concentration	3×10^{-5} *M*	84 (3)		
	1×10^{-4} *M*	56 (3)		
	1×10^{-3} *M*	25 (3)		
Iodoacetate				
Final concentration	1×10^{-3} *M*	3 (2)		
P.C. solution				
Incubation time	1 hr.	75 (10)		
Incubation time	4 hr.	258 (2)		
P.C. solution with glucose				
Incubation time	1 hr.	99 (10)		
Incubation time	4 hr.	422 (2)		

* The data are given as means of cts/sec/mg N for the number of determinations indicated by the figures in parenthesis.

† The per cent values refer to the fraction of tracer incorporated after removal of the epithelium.

tion was found to be greatly reduced. In the absence of the epithelium the uptake of glycine-1-C^{14} into the I.S.F. of the corneal stroma of the embryos and of the hatched chick was found to be only 15 and 5 per cent, respectively, of the tracer incorporation into the I.S.F. of the stroma observed in the presence of the intact, unseparated epithelium. On removing one-half or three-quarters of the epithelium, the uptake of tracer into the stroma seems to fall approximately in proportion to the amount removed. It should be mentioned at this point that the addition of separated sheets of epithelium to the stripped stroma does not reverse the effect of the separation of the two tissue components. The separated epithelium also shows a considerable decline in tracer incorporation into the epithelial proteins (300–400 cts/sec/mg N *in situ;* 40–60 cts/sec/mg N after removal). This test was carried out with epithelia from hatched chick since in this case the epithelia can be removed as large sheets with minimal cell disruption.

In one experiment six corneas each from 7-day chick were incubated with and without epithelium. An aliquot of the I.S.F. was hydrolyzed at 140° C. for 4 hours. The glycine of this fraction was separated chromatographically as 1-fluoro-2,4-dinitrobenzene derivative, following essentially the method of Krol,[8] and the specific activity of the glycine derivative was determined. Calculated for a single cornea, a glycine content of 61 μg. and of 55 μg. was found for the I.S.F. of the stroma incubated with and without epithelium, respectively. The corresponding activities were 423 cts/min and 62 cts/min, giving specific activities of 6.9 cts/min/μg glycine and 1.1 cts/min/μg glycine, respectively. This experiment shows that the observed dif-

ference in the specific activities of the I.S.F. is due to the different specific activities in the protein glycine of this fraction. The glycine content of the I.S.F. found in this experiment is about twice the hydroxyproline concentration determined[9] for the same fraction. The fact that the glycine : hydroxyproline ratio is 2 is consistent with the view that this protein is the predominant component of the I.S.F., though it does not exclude the possibility that other proteins, having little or no glycine, may also be present.

The tracer uptake into the I.S.F. fraction of the stroma of the intact cornea increases continuously at least for a period of 6 hours. In the embryonic cornea a slight acceleration of the incorporation rate was observed which was absent in the corneas of hatched chicks. The dependence of the stroma incorporation of metabolic energy is indicated by its sensitivity toward dinitrophenol and iodoacetate. But the addition of 1 mg. ATP to the medium did not increase the incorporation of the stripped stroma. The cornea can apparently utilize endogenous metabolites as sources of energy and as protein precursors, since replacement of the complete medium by a solution made up of inorganic constituents (P.C. solution[10]) still allows incorporation to take place, although at a diminished rate. It remains to be seen whether the tracer uptake in the absence of external energy sources actually indicates complete protein synthesis or is related to "exchange" reactions observed in bacteria.[11] Considerable stores of glycogen which were depleted during maintenance *in vitro* were demonstrated in the beef cornea on a previous occasion[12] and the presence of similar reserves of metabolites in the chick cornea would seem to be likely. The addition of the glucose to the inorganic medium increases the incorporation rate significantly.

Discussion.—The drastic reduction in the protein formation after separation of the tissue components of the chick cornea raises the question of the mechanism by which the epithelial and stroma cells maintain optimal protein formation in the intact cornea. In the case of the epithelium, it seems possible that even very careful removal would lead to injury of the cells. Certainly some effect on the electron-microscopically distinct[13] epithelial cell surface adhering to the stroma has to be considered. Whether this disturbance is sufficient to lead to the phenomenon of injury metabolism which has been observed in some tissues,[14,15] but not in others,[16] has not been decided so far. Tests of protein formation in the cornea after a chemical separation of the epithelium and stroma (trypsin, versene) are now in progress. In this context, it is of interest that iodoacetate, which completely inhibits the incorporation of the tracer into the stroma, also leads to a complete loss of cohesion of the epithelium.[17]

In the case of the stroma, cell injury as a cause of metabolic disturbance would seem rather unlikely, since these cells are imbedded in a matrix of ground substance which increases in mechanical strength with the progress of development. In the hatched chick the stroma surface is of leathery toughness because of its collagen content. But just here a more complete inhibition of protein formation was observed. As an alternative, it can be considered that the metabolic energy necessary for protein formation in the stroma is, at least to a greater part, supplied by the metabolism of the epithelium. The fact that in beef corneas almost the entire oxidative metabolism occurs in the epithelium would make such a possibility plausible,[12, 18] though, of course, not a universal condition for collagen biosynthesis (e.g., in tissue cultures of connective tissue).

Functionally important metabolic interactions between ectodermal and mesodermal tissue constituents have been described previously by other investigators.[19,20] The existence of a similar interaction for the maintenance of protein formation in cells with relatively low oxidative metabolism by another cell type with a different metabolic pattern could be of significance as a form of cell interaction occurring during embryonic development. Experiments designed to explore more extensively such a possibility are in progress at this laboratory.

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[1] Heinz Herrmann, Leonard Lerman, and Betty White, *Biochim. et Biophys. Acta* (in press).
[2] Heinz Herrmann and Phyllis W. Schultz, *Arch. Biochem. and Biophys.* (in press).
[3] Phyllis W. Schultz and Heinz Herrmann, *J. Embryol. and Exptl. Morphol.* (in press).
[4] Charles I. Thomas, *The Cornea* (Springfield, Ill.: Charles C Thomas, 1955).
[5] Phillip I. Marcus, Steven J. Cieciura, and Theodore T. Puck, *J. Exptl. Med.*, **104**, 615, 1956.
[6] O. H. Lowry, D. R. Gilligan, and E. M. Katersky, *J. Biol. Chem.*, **139**, 795, 1941.
[7] Heinz Herrmann and Shirley R. Barry, *Arch. Biochem. and Biophys.*, **55**, 526, 1955.
[8] S. Krol, *Biochem. J.*, **52**, 227, 1952.
[9] Heinz Herrmann, unpublished data.
[10] Charles A. Pannett and Arthur Compton, *Lancet*, **206**, 381, 1924.
[11] E. F. Gale and John P. Folkes, *Biochem. J.*, **55**, 721, 1953.
[12] Heinz Herrmann and Fay H. Hickman, *Bull. Johns Hopkins Hosp.*, **82**, 251, 1948.
[13] Huntington Sheldon, *J. Biophys. Biochem. Cytol.*, **2**, 253, 1956.
[14] Otto Warburn, Karl Posener, and Erwin Nägelein, *Biochem. Z.*, **152**, 309, 1924.
[15] N. Brock, H. Druckrey, and H. Herken, *Biochem. Z.*, **300**, 1, 1939.
[16] Frank Dickens and Hans Weil-Malherbe, *Biochem. J.*, **35**, 1, 1941.
[17] Heinz Herrmann and Fay H. Hickman, *Bull. Johns Hopkins Hosp.*, **82**, 182, 1948.
[18] Heinz Herrmann and Fay H. Hickman, *ibid.*, p. 260.
[19] Louis B. Flexner and Robert D. Stiehler, *J. Biol. Chem.*, **126**, 619, 1938.
[20] Jonas S. Friedenwald, Wilhelm Buschke, and Harry O. Michel, *Arch. Ophthalmol.*, **29**, 535, 1943.

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*SPECTRAL LUMINOSITY CURVES FOR PROTANOPIC, DEUTERANOPIC, AND NORMAL SUBJECTS**

BY YUN HSIA AND C. H. GRAHAM

DEPARTMENT OF PSYCHOLOGY, COLUMBIA UNIVERSITY

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The luminosity curve shows how the sensitivity[1] of the eye varies for various wave lengths. Sensitivity is measured in terms of the reciprocal of the energy required to produce a given effect—threshold, for example.

In 1952 we published an article on the foveal luminosity curves of five normal subjects.[2] The present account is concerned with the spectral sensitivity curves of two groups of color-blind subjects,[3] five protanopes and six deuteranopes, as compared with the curves obtained on seven normal subjects. Until the work of Hecht

and Hsia[4] it was not considered that deuteranopes show a loss of cone sensitivity, as compared with normal subjects, in any part of the spectrum. Hecht and Hsia found, on calculating sensitivities based on the computed energies of their filtered lights, that deuteranopes seem to show a loss of sensitivity in the green region of the spectrum. They emphasized the fact that some earlier results which seemed to show no loss of green sensitivity in deuteranopes could be due to the prevalent practice of plotting per cent values of sensitivity rather than values based on energy.[5]

The present experiments have been performed to investigate, by improved techniques, the still not universally accepted fact of cone luminosity losses in deuteranopes. We have also obtained threshold luminosity data on a small group of protanopes. Our results show that, as compared with normal subjects, both types of color-blind individuals demonstrate sensitivity losses; five of the six deuteranopes show a deficit of luminosity in the green-to-blue part of the spectrum, and all the protanopes show a loss in the red.

Subjects.—For our measurements we used five protanopes and six deuteranopes. Our seven normal subjects consisted of the five whose foveal data have been described earlier[2] plus two additional ones, a man and a woman, each about twenty-five years of age.

The testing of all subjects involved the reading of the Ishihara and Stilling plates, the determination of the proportion of red and green required to match a yellow on the Hecht-Shlaer anomaloscope,[6] and the determination of the neutral point in the spectrum. The tests are all highly discriminative for the three classes of individuals studied—normal, protanopic, and deuteranopic.

The color-blind subjects were all complete dichromats. We first chose those persons who made extensive errors in the Ishihara and Stilling tests. Of these, we selected only those who in the anomaloscope were able to match perfectly the yellow of 575 mμ with a green of 555 mμ by itself and also with the red of 635 mμ by itself. By comparing the relative luminances at match of the yellow and red, we classified the subject as protanopic or deuteranopic. If the red luminance required by a subject was disproportionately greater (to a normal eye) than the yellow, that subject was called a protanope, whereas if, at match, the red and yellow were approximately equal, he was called a deuteranope. The final critical test was always to determine the presence in the spectrum of a neutral point (the narrow wavelength band seen as white by dichromats). Only those subjects who matched a sharply located neutral point with white light of 5,000° K. as a standard were considered to be complete dichromats. The neutral-point determinations were made with a modified Helmholtz color mixer.[7]

Apparatus and Procedure.—Our observations involved the use of a double monochromator whose light could be varied by the use of appropriate controls with respect to wave length, energy, duration, and subject's retinal position. The energy measurements were done with a photronic cell (Weston, Model 594 RR, at the pupil position) and a moving-coil galvanometer. The apparatus, essentially a Maxwellian view system, was in all details similar to the one described earlier.[2]

We found the subject's threshold at each wave length tested. Our test field was a circular one subtending a visual angle 42 minutes of arc in diameter. The test field was viewed by the subject's dark-adapted eye and appeared in the center of a fixation ring, i.e., a thin, dim ring of white light that subtended a visual angle 2.5°

in diameter. The duration of each single stimulus exposure was 4 milliseconds.

At the beginning of each session, the subject was dark-adapted for 10 minutes, presumably a sufficient time for the cones to gain full sensitivity. The subject presented each spectral light to himself by releasing a key. Presentations were so arranged that the light stimulations were in accord with the method of limits, three ascending and three descending steries being given for each threshold determination. Three such thresholds, each one on a different day, usually provided the final data for the subject's average threshold at a given wave length.

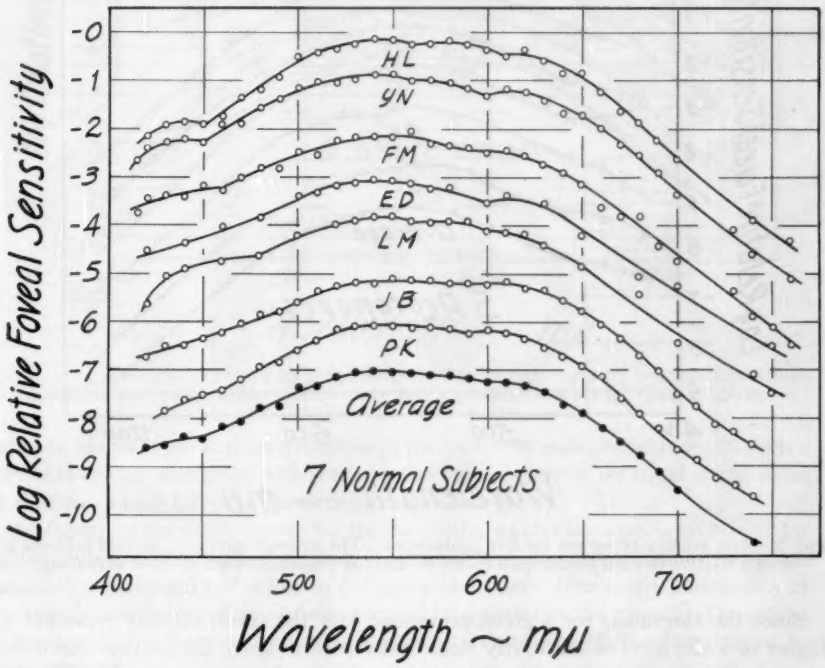

FIG. 1.—Log sensitivity curves as a function of wave length for seven normal subjects. The average curve is given at the bottom of the figure.

Results on Normal Subjects.—Figure 1 gives the individual log sensitivity curves for our seven normal subjects; the bottom curve presents the averaged data.[8] The basic data of these curves are relative energies required for the cones to respond to the spectral lights at the absolute threshold; the logarithms of the reciprocals of these values (i.e., log sensitivity values) are here plotted. The peak of the average curve is arbitrarily set at zero (i.e., maximum sensitivity is set at unity). For purposes of clarity of presentation, each successive curve is moved downward through one logarithmic unit. The absolute energies may be computed by observing that at 578 mμ the average normal threshold (interpolated) is 3.5×10^{-8} erg, or about 10,000 quanta.

It is probably not necessary to labor the fact of the "humps" in the blue and in the orange, near 450 and 610 mμ. Presumably in the normal subject they are manifestations of three (or more) primary processes.

Results for the Protanopes.—Figure 2 gives the luminosity curves for five protanopes. Each curve represents the data for an individual, and the average curve is given at the bottom of the graph.

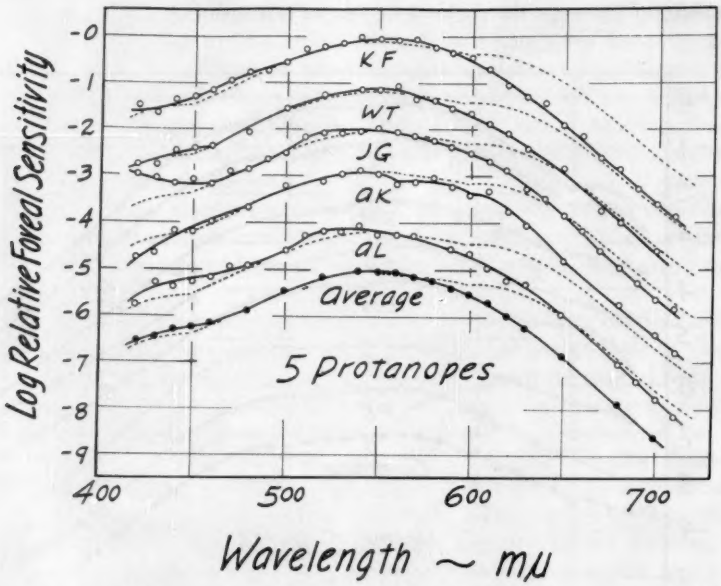

FIG. 2.—Log sensitivity curves for five protanopes. The average curve for normal subjects is drawn relative to each protanope's curve, so that log sensitivities are equated at 500 mμ.

Since the thresholds for a given protanope over the spectrum may represent a higher or lower level of sensitivity than that represented by the average curve for normal subjects, it is desirable that each protanope be compared, not with the average normal subject, but rather with a normal subject whose sensitivities are, as it were, equivalent in those regions of the spectrum not affected by the condition of protanopia. On this basis the average curve for normal subjects has been drawn (*dotted lines*) relative to the curve for each protanope, the curve for normal subjects being so placed on the ordinate axis that the value of the log sensitivity at 500 mμ is set equal to the value lying on the curve for each protanope at that wave length.

It is to be observed that the curve for each protanope indicates a greatly increased energy requirement in the red part of the spectrum. The protanope loses luminosity at long wave lengths.

Results for the Deuteranopes.—Figure 3 gives the results for the deuteranopes. Each single curve is the luminosity function for a single deuteranope. The average curve is presented at the bottom of the graph. The average curve for the normal

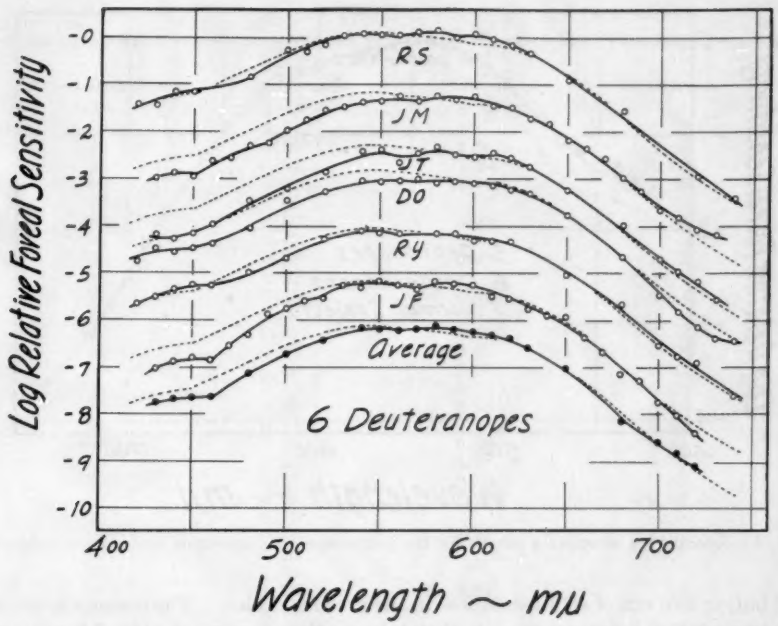

FIG 3.—Log sensitivity curve for six deuteranopes. The average curve for normal subjects is so placed relative to each deuteranope's curve that log sensitivities are set equal at 650 mμ.

subjects (*dotted lines*) is drawn relative to the curve for each deuteranope in such a way that the log sensitivity at 650 mμ for the normal curve is set equal to the value at that wave length for the given deuteranopic curve.

As shown by the single curves for the deuteranopes and the associated curves for normal subjects, it would seem that, in general, the deuteranope shows a normal luminosity in the red but a loss in the green and blue. (On an arithmetic plot of sensitivity, the degree of absolute loss would be greatest in the green.) The extent of the loss varies from subject to subject, and in one case (RS) a loss cannot be demonstrated.

The existence of the single deuteranope who, by our standards, does not show a significant loss may raise a question: Does the loss that seems to be usual in deuteranopia, as shown by its existence in five of our six subjects, exhibit a determinative character, or is it a correlated but not causative condition? We are in no position to discuss this matter and will only repeat what we have said previously: luminosity loss seems to be a usual condition in deuteranopia.

Summary of Results for All Groups.—Figure 4 and Table 1 summarize the average data for normal subjects as well as for the protanopes and the deuteranopes. The data, as presented in Figure 4, directly represent average log sensitivity values for all groups; no arbitrary adjustments along the ordinate axis have been made.

In general, one can say that the normal curve is a broad function that encompasses the extremes of the color-blind curves. Protanopes have nearly normal luminosity in the blue but show loss in the red; deuteranopes show normality in the

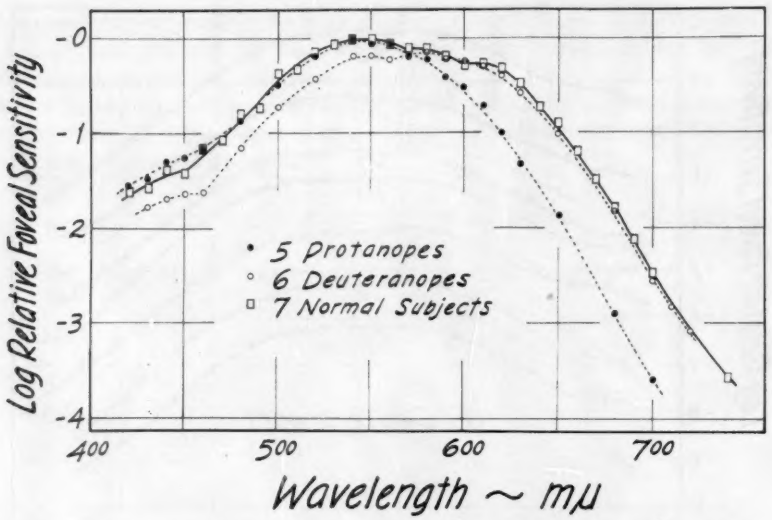

FIG. 4.—Average log sensitivity curves for the protanopes, deuteranopes, and normal subjects.

red but, in five out of six cases, a loss in the green and blue. The manner in which the two average functions cross as they take up their regions of normal luminosity provides an interesting picture; the protanope loses luminosity in the spectral region, where the deuteranope shows normal sensitivity. Conversely, the deuteranope loses sensitivity in the region where the protanope is normal.

The average curve for protanopes appears to be above the curve for normal subjects in the blue part of the spectrum. This apparent increase in luminosity is almost certainly not real; it depends almost completely on a single subject, JG, whose luminosities in the blue seem relatively high.

Humps in various luminosity curves might be expected to appear more clearly in individual curves than in averaged data. It is nevertheless true that the normal curve of Figure 4 shows a well-marked hump near 450 mμ and probably another in the region near 575 to 600 mμ. The latter hump seems to be definitely smoothed out in the curve for the protanopes, and the green peak near 540 mμ is diminished for the deuteranopes. The blue hump is, relatively, not conspicuously affected in the latter case.

Discussion.—Through the years since the time of Young,[9] it has been supposed that protanopia is due to the loss of what we may call R-receptors, those that are especially sensitive to red. On the other hand, it has not generally been accepted that deuteranopia is due to a loss of G-receptors, those that are especially sensitive to green.

Because the evidence did not seem to support an interpretation of loss for the deuteranope, another sort of account seemed to be required for this type of color defect. The most widely held theory of deuteranopia has entailed the idea of a "transformation" system, as first described by Leber[10] and by Fick.[11] One can think of the transformation system as representing a failure of the R- and G-receptors to

TABLE 1*

AVERAGE LOG_{10} RELATIVE ENERGY VALUES FOR SEVEN NORMAL, FIVE PROTANOPIC, AND SIX DEUTERANOPIC SUBJECTS

Wave Length (Mμ)	Normal Subjects	Protanopes	Deuteranopes
420	1.61	1.53	..
30	1.57	1.47	1.77
40	1.39	1.29	1.68
50	1.42	1.24	1.63
60	1.17	1.16	1.62
70	1.07	..	..
80	0.80	0.85	1.15
90	0.73	..	..
500	0.38	0.48	0.72
10	0.33	..	..
20	0.15	0.17	0.44
30	0.07	..	..
40	0.01	0.02	0.19
50	0.02	0.07	0.18
60	0.06	0.05	0.22
70	0.11	0.18	0.20
80	0.12	0.22	0.14
90	0.19	0.41	0.22
600	0.28	0.53	0.24
10	0.27	0.71	0.32
20	0.32	1.00	0.40
30	0.48	1.27	0.58
40	0.73	..	..
50	0.89	1.87	1.01
60	1.20	..	..
70	1.50	..	..
80	1.79	2.91	1.83
90	2.13	..	..
700	2.49	3.60	2.58
10	..	..	2.83
20	..	..	3.10
30	..	..	..
40	3.58	..	..
50	..	..	..
60	..	..	..

* Concerning the data of individuals, see fn. 8. The absolute energy value for the average normal subject 578 mμ (interpolated) is 3.5×10^{-8} erg, equivalent to about 10,000 quanta at the cornea.

become differentiated from each other. They are presumed to have similar absorption characteristics but different central connections. The result would be that red, for example, would stimulate both central R- and G-systems. Since R and G produce yellow, it would be expected that the deuteranope would call all long wave lengths yellow, a response that is, in fact, in accord with what a unilaterally deuteranopic person sees in his color-blind eye: all long wave lengths are yellow. This yellow can be compared with what is seen in the normal eye.[12]

It must be understood that the transformation theory is applicable to protanopia as well as to deuteranopia. It has not been applied in the former case because of the fact that most workers seem to have accepted the principle of luminosity loss in protanopia. However, it should be noted that the narrowing of the classically drawn luminosity curve in protanopia could just as well be taken to be due to a "transformation" shift in absorption of the R-receptors toward that of the G-receptors.

The present result and that of Hecht and Hsia[4] raise problems for the Leber-Fick type of theory.[10, 11] There is a loss of green luminosity in a manner inconsistent with the latter theory. On the other hand, if we accept the fact of loss, how can

we account for a deuteranope's seeing of yellow, when, instead of G's absorption characteristics being "transformed" to those of R, G-receptors are lost, at least to a considerable extent?[13]

The present experiment gives no answer to the problem. A next step in analysis may profitably involve research on a unilaterally dichromatic subject; for, in the past, data on such subjects have not usually been extensive. We have been fortunate to obtain the services of a unilaterally dichromatic subject with a deficiency of spectral sensitivity primarily in the green. Reports of experiments on this subject will appear soon.

Summary.—Data have been obtained on three groups of subjects, made up, respectively, of five protanopes, six deuteranopes, and seven normal subjects. As compared with normal subjects, protanopes show a loss of luminosity in the red. In contrast with some previous interpretations but in conformity with the results of Hecht and Hsia,[4] five of the six deuteranopes show a loss of luminosity in the green.

* This work was supported by a contract between the Office of Naval Research and Columbia University and by a grant-in-aid from the Higgins Fund of Columbia University. Reproduction in whole or in part is permitted for any purpose of the United States government.

Preliminary accounts in abstract form of the present experiment have appeared in C. H. Graham and Y. Hsia, *Proc. Fourteenth Intern. Congr. Psychol.*, p. 114, 1954; also *Science*, **120**, 780, 1954.

We are indebted to Professor Lucy J. Hayner, of the Physics Department, Columbia University, and Dr. Simon Shlaer, of the University of California, for technical discussions concerning our equipment.

[1] The terms *sensitivity* and *luminosity* as here used are synonymous with each other and with the terms *luminous efficiency* and *visibility*.

[2] Y. Hsia, and C. H. Graham, these PROCEEDINGS, **38**, 80, 1952.

[3] Protanopes and deuteranopes are two classes in a larger class called "dichromats." The class of dichromats comprises, in addition to protanopes and deuteranopes, people in a rare class called "tritanopes" and those in a still rarer class called "tetartanopes." Dichromats can match any color of the spectrum by a mixture of two colors. Protanopes confuse red and green and show a loss of sensitivity in the red part of the spectrum. Deuteranopes also confuse red and green but require amounts of these two colors to match yellow that are different from those required by the protanopes. Tritanopes confuse blue and green. Tetartanopes also exhibit confusions between blue and green, but, unlike the other dichromats, who match a narrow region of the spectrum with white, the tetartanopes match two regions of the spectrum with white.

[4] S. Hecht and Y. Hsia, *J. Gen. Physiol.* **31**, 141, 1947.

[5] For example, see F. H. G. Pitt, *Great Britain Med. Research Council Special Rept. Ser.*, No. 200, 1935.

[6] For a discussion of the Hecht-Shlaer anomaloscope see M. P. Willis and D. Farnsworth, *Med. Research Lab., U.S. Naval Submarine Base*, Vol. 11, No. 7, Rept. No. 190, 1952. References to the Ishihara and Stilling tests are as follows: S. Ishihara, *Tests for Colour-Blindness* (6th ed.; Tokyo: Kanehara, 1932); E. Hertel, *Stillings pseudo-isochromatische Tafeln* (Leipzig: Georg Thieme, 1936).

[7] S. Hecht and S. Shlaer, *J. Gen. Physiol.*, **20**, 57, 1936.

[8] The numerical data of individual subjects, color-blind and normal, have been deposited with the American Documentation Institute (order Doc. No. 5366 from ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D.C., remitting in advance $1.25 for microfilm or $1.25 for photocopies. Make checks payable to Chief, Photoduplication Service, Library of Congress.

[9] T. Young, "On the Theory of Light and Colours," in *Lectures in Natural Philosophy*, **2** (London: Joseph Johnson, 1807), 613.

[10] T. Leber, *Arch. Ophthalmol.*, **15** (3), 26, 1869.

[11] A. Fick, "Die Lehre von der Lichtempfindung," in *Handbuch der Physiologie*, ed. L. Herrmann, **3**, Part 1 (Leipzig: Vogel, 1879), 139.

[12] See, on the topic of unilaterally color-blind persons, the discussion by D. B. Judd, *J. Research Nat. Bur. Standards*, **41**, 247, 1948.

[13] For strong objections to the idea of luminosity loss in deuteranopia see G. L. Walls, and R. W. Matthews, *Univ. Calif. Publs. Psychol.*, Vol. **7**, 1952.

ON SOME PHENOMENA IN DROSOPHILA RELATED TO SO-CALLED GENIC CONVERSION

BY RICHARD B. GOLDSCHMIDT

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY

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While analyzing the action of the rudimetary locus (r, 1, 54, 5),[1] many backcrosses were made involving one X-chromosome marked with Bar (B, 1, 57.0), with or without additional markers, and the rudimentary alleles r^9 and r^{39}, the latter carrying also Bar and forked (f, 1, 56.7). In view of the ± sterility of rr females, the backcrosses were always (B/B ♀ × r♂) × r♂, which gives $^1/_2$ females rr, $^1/_2$ females +B/r+, $^1/_2$ males B, $^1/_2$ males r. The variations of this general scheme were the use of r^9 or r^{39} f B males. All combinations were made, viz., (B × r^9) × r^9, (B × r^9) r^{39}, (B × r^{39}) r^9, (B × r^{39}) r^{39}. In addition, five different chromosomes with B were used, three of them kept over different crossover inhibitors. The unexpected results we obtained in 6 out of more than 400 R F_2, 5 in (B^{St} × r^9) × r^{39} 1 in (B^{Pas} × r^9) r^9. (The number of normal backcrosses has increased considerably since, without a repetition of the unexpected findings.)

The First Group.—From the Bar stock which we designate as B^{St} (not containing crossover inhibitors) single females were crossed to r^9 males and eleven F_1 females, partly sisters, partly not, backcrossed individually to r^9 males. All results were as expected. Eight more females of the same provenience were backcrossed to r^{39} f B males. The results of three matings were again according to expectation (see Table 1). The somewhat irregular crossover classes may be chance happenings. The over-all value of 3.5 per cent crossovers is about normal (2.5 map distance

TABLE 1

RF_2 (B^{St} × r^9) × r^{39} f B PHENOTYPES

	♀			♂			
No.	B/B	B/+ r/r	B/B r/r	B	r	rB	+
4572	101	91	3	62	42	19	1
4573	54	69	1	59	52		1
4574	78	72	4	71	70		1
S.	233	232	8	192	164	19	3
4566	66		45	16		40	
4568	43		54	43		40	
4569	39	10	40	31	3	34	
4570	54	4	39	51	5	43	
4571	71	2	76	60		64	
S.	273	16	254	201	8	224	

r → B). The apparent absence of one female crossover class may be due to chance. The remaining five backcrosses had an aberrant, but completely consistent, result. As the bottle numbers recorded in the first column indicate, all these aberrant results come from mothers who were sisters, which is important.

Daughters and sons showed the phenotypes expected if the mother had been ++B/r+B, namely, females ++B/rfB = phenotype B/B and r+B/rfB = phenotype rB/rB; further males ++B and r+B. In addition, three of the five broods contained small classes phenotypically B/+ rr females and r males.

In Table 2 the total results are given with the genotypic constitution of the classes. The first group contains the normally behaving crosses with the four classes of expected females and males and the four crossover classes, one of them missing, one abnormally large. In the second group we find the totals of the abnormal crosses with the four classes of females and males expected if the mother had been ++B/r+B. The two additional classes, looking like crossover classes, are called "unexpected" because they cannot be derived by simple crossing-over from ++B/r+B mothers. The phenotypes of these unexpected individuals are, most importantly, those of the two noncrossover classes if the mother had been ++B/r++ and had segregated normally! Assuming that these unexpected classes were produced by some unexplained crossing-over, one would expect two reciprocal crossover classes of the same phenotype as the two other noncrossover classes in the normal case, i.e., females ++B/rfB and males ++B. Since both these types are also present in the abnormal case as two of the four noncrossover types, the expected small number of reciprocal "crossovers" of these two types can be contained indistinguishably in those two large classes.

Now, if we desregard for a while the crossover-like small groups, the production of gametes—leaving out forked, which does not show in the females and is absent in the males—$^1/_2$ B, $^1/_2$ rB by a heterozygote +B/r+ requires crossing-over of B into the r-chromosome and, in addition, the retention of B in the original chromosome. Such an apparently impossible happening could also be described as conversion of $+^B$ in the r-chromosome into B under the influence of the B in the homologue. Here is now a chance to find out something about so-called *conversion*, because of the fact that we know that B has a tendency to unequal crossing-over (Sturtevant) and that B is actually a tandem duplication of a chromosome segment and BB (double Bar, produced by unequal crossing-over) a triplication (Muller, Bridges). This situation will permit us to show what the "conversion" really is, in this case, and might be in others.

TABLE 2

TABLE 1 ARRANGED FOR EXPECTED AND UNEXPECTED GENOTYPES IF MOTHERS WERE ++B/r++ OR ++B/r+B

	Expected				Crossover			
	♀ ++B/rfB	♀ r++/rfB	♂ ++B	♂ r++	♀ r+B/rfB	♀ +++/rfB	♂ r+B	♂ +++
Mother ++B/r++	233	232	192	164	8	...	19	3
	Expected				Unexpected			
	♀ ++B/rfB	♀ r+B/rfB	♂ ++B	♂ r+B	♀ r++/rfB	♀ ++B/rfB	♂ r++	♂ ++BB
Mother ++B/r+B	273	254	201	224	16	?	8	?

We had noticed that the B^{St} stock sometimes contained + flies, which means a tendency to unequal crossing-over, producing double Bar and normal flies. Double Bar had not been seen in the stock but is easily overlooked if heterozygous with B and present only in small numbers in a stock. Thus it is possible that an exceptional BB/B female (BB = double Bar, B/B = homozygous Bar, etc.) was used for the first cross meant to be B/B × r. The phenotype of this compound is not outside the range of variation of that of B/B and therefore could have remained unnoticed if not looked for. Thus, among many F_1-sisters used for the backcrosses, both types would have been present in equal numbers, +B/r+ and +BB/r+. This entails at once a chance for the production of B and rB gametes, namely, by the unequal crossing-over of one B segment of BB into the r-chromosome which can also be described as a crossover break between the second and third Bar segment after synapsis of the + segment with the second segment of BB. Now the aberrant fact is that this unequal crossing-over must have occurred in all gametes (still disregarding the few apparent crossovers). A 100 per cent unequal crossing-over in all gametes is utterly improbable, as all previous work indicates that unequal crossing-over occurs only when crossing-over is established between near-by markers, i.e., in a few crossover gametes. Unfortunately, in our experiment there is no marker to the right of B, which would allow us to pin down what had happened. It is still more improbable that this 100 per cent unequal crossing-over occurs in five different sister broods without some condition different from the usual case. As the absence of a marker near B on its right prevents a definite statement about the presence or absence of a rather improbable genuine crossover occurrence, unequal for the Bar triplication, we can only state the possibilities of an explanation.

First, the unexpected happenings occurred in five sister broods, three others being normal. This requires the assumption that the BB chromosome present in all abnormal broods contained an unusual and very rare genetic condition (heterozygous in the P-♀) which causes the abnormal phenomenon. This conclusion is nothing unusual if we think of the work in maize (Rhoades, McClintock) with the loci Dt and Ac of comparable function. Any explanation of the 100 per cent unequal crossing-over will require such a controlling dominant factor, which we shall call a *transductor*.

Second, it is possible that the gametes B and rB are produced at the meiotic divisions by unequal crossing-over from BB. In this case, it cannot have happened at the four-strand stage because only two major classes of gametes were formed. Further, it cannot have happened together with ordinary crossing-over, which could entail unequal crossing-over only in a small fraction of the gametes. Therefore, the unequal crossing-over must have taken place in a two-strand stage; and further it must have taken place in every single egg cell under the influence of the dominant factor discussed in the first paragraph.

Third, it might be possible to account for the result if this unequal crossing-over had already occurred in one single cell of the developing F_1 female, namely, a cell of the germ track from which all gametes are derived. As almost all gametes are B and rB, this somatic unequal crossing-over must again have taken place between one-strand homologous chromosomes of the stem cell of the germ track. The identical behavior of all five abnormal females requires, in addition, the presence of

the dominant transductor in all F_1 females with abnormal offspring. This explanation would require the not very appealing assumption that the unequal crossing-over takes place in the prophase of the first (or one of the first) segmentation divisions between not yet divided homologous chromosomes. But the possibility cannot be excluded.

Fourth, it is possible that what appears to be unequal crossing-over is no crossing-over in the classic sense at all. After all we know about the happenings in maize, there is no reason why the locus responsible for the phenomenon, which we compared with Dt, etc., in maize, should not act as a real transductor. This means that in its presence one of the three Bar segments of the BB-chromosome is lifted out and transduced (in the exact sense of this term as established in bacteria) into the homologue in the two-strand stage. This is the same event as has been called, in terms of crossing-over, "negative interference" in some cases of conversion in plants. This idea involves the need for an explanation in classic cross-over terms under all circumstances. I personally think that this assumption of genuine transduction will finally turn out to be correct and that it will easily explain all the cases of so-called *conversion*.

It is very regrettable that in the present experiment no markers were present which permitted a definite statement. It is still more regrettable that the presence of what we may now call a transductor locus was not immediately suspected and the chromosome containing it isolated. Thus a chance recurrence in a properly made-up cross must be awaited for a final decision. Many hundreds of backcrosses made since were normal expect one, which occurred soon after the present experiment and which is still more difficult to analyze (see below).

We return now to the small classes in the abnormal set of backcrosses, which suggest crossover classes and the phenotype of which is the same as that of one of the noncrossover classes (in either sex) in the normal backcrosses. The phenotype of the reciprocal crossover class could, if present, be indistinguishable from that of one of the large classes. Thus it is probable that we are dealing with crossover classes, though it cannot be proved. (see Table 2, unexpected classes). This suggests at once the idea that the dominant transductor locus, which we postulated before, is present in the BB chromosome and could be removed from it by crossing-over. If this locus acts only upon BB (to produce unequal crossing-over or else transduction), when located in the same chromosome the crossovers for the transductor would be the unchanged maternal chromosomes BB and r. Actually, no BB males were registered, but the possibility cannot be excluded that a very few of them, not looked for as they were, were overlooked among the 200 B males. If this is the correct explanation for the apparent crossover class—and it can hardly be otherwise—it would follow that the unequal crossing-over (or transduction) in the triplication would have had to take place after meiosis. But it should be added that we have no final proof that the small class is really a crossover class. As it is genetically identical with the noncrossover classes in the normal segregation, the possibility remains that for some unknown reason in a few cases the production of abnormal gametes B and rB has not taken place. In the absence of other markers, this cannot be decided. It is most disappointing that the whole phenomenon is thus far only an uncontrollable rarity, which did not permit a full analysis. But even at this stage we have reason to visualize an explanation of so-called

conversion in terms of transduction of duplicated or triplicated (or even pseudo-allelic) chromosomal segments under the control of a dominant transductor.

The Second Group.—The thus far uncontrollable genetic conditions have produced a single other comparable instance complicated by additional features which could not be completely analyzed. But the facts are so interesting that we feel justified in announcing them, especially as it cannot be predicted that they will occur again under better conditions for analysis. This second case was found in one out of nineteen identical backcrosses $(B^{Pas} \times r^9)r^9$. This means that eighteen times the expected segregation into +B/r+ and r/r females and B and r males occurred. One backcross (4842) gave the following result, which cannot be explained away as experimental error:

♀ Phenotypes	♂ Phenotypes
87 between B/B and B/+	72 typical double Bar
79 rr	78 r
1 +	1 +
1 like rB/r+	2 barlike, but different (smaller eye)
	2 typical infrabar and rudimentary

It is obvious that the F_1 mother had not been +B/r+ but +BB/r+ (heterozygous double Bar), which is within the range of variation of +B/r+ and could easily have remained unnoticed. Whereas, if the grandmothers of the set of 19 backcrosses had been B/B, the unequal crossing-over to BB must have occurred in the meiosis of one grandmother, which led to a few F_1 females +BB/r+, one of which was picked out for the backcross by chance. The big classes in RF_2 are exactly what is expected in this case. (The BB males were tested and extracted and BB/BB females extracted in F_3 in the typical way.)

We now turn to the crossover classes. Among the females were one each of the expected crossovers, namely, eggs r → BB = rBB and ++, which, mated with r males, gave the crossover classes ++/r+ and rBB/r+. In the males, only one of the expected crossover classes was present, namely, one male +. In addition, there were two males nearly Bar (registeres as B? because the eyes were atypical, though resembling Bar) and two males typically infrabar and rudimentary. The latter bred true with *X* females and also sired homozygous infrabar females with rough eyes, according to expectation.

Thus infrabar must have been produced by unequal crossing-over of some section of the BB triplication into the homologue with r. The simultaneous finding of two males similar to, but not identical with, B(B?) suggests a reciprocal class to Bi and makes it improbable that Bi appeared as a mutant in the r-chromosome. The appearance of Bi in a situation very similar to that of the original occurrence in Sturtevant's classic work is strongly in favor of such an interpretation. (Of course, no reciprocal class was noted then, and Bi was described as an ordinary mutant in the homologue.) This means that Bi and B(?) must be the products of unequal crossing-over from the BB into the r chromosome, different from an exchange of a whole Bar segment in classic unequal crossing-over, with the result of $+\,\big|\,{}^{B?}_{Bi}$ r instead of $+\,\big|\,{}^{B}_{B}$ r. As also one + male appeared in the same RF_2, both normal and unequal crossing-over had taken place, and the latter in an unusual way, producing Bi and B?. This could mean a break inside the third Bar segment instead of between the

second and third segment. Thus part of the third segment remained in place and the two segments, plus this part, produced the B? phenotype, while the rest of the third segment added to the normal segment (+B) becomes the constitution of Bi. It is generally assumed that the salivary structure of infrabar has not been established definitely, though it has been claimed to be identical with that of Bar. If this were approximately true, meaning that only a small, thus far undetected, difference exists, we would expect B? to be almost identical with B, while Bi would have some small part of the B section added as a part duplication. It is most unfortunate that the B? males did not sire any offspring with attached X-females and that, in the rush of work, the new Bi was discarded after some generations of testing for breeding true before I realized the importance of keeping it for salivary studies. Thus the foregoing interpretation remains hypothetical, though, in my opinion, highly probable, until a repetition occurs in a properly marked experiment, with a chance to isolate the B? partner.

The RF_2 females, which looked more like B/B than like B/+ and had to be assumed to be +BB/r+, were bred, and it turned out that we faced another cass of 100 per cent unequal crossing-over, but in another generation than in the firet case. A number of the RF_2 females which would be +BB/r+ were mated to both BB and r males. The result was that a number of them produced the expected offspring, i.e., BB and r sons, and also the expected daughters in each case, as well as a few typical crossovers. But two others of these females produced sons $^1/_2$ BB, $^1/_2$ r Bi and, if the father was BB, daughters $^1/_2$ BB/BB and $^1/_2$ like the mothers. Thus part of the F_2 females which were all assumed to be +BB/r+ and could hardly contain two different genotypes, had produced half males classified as BB and half r Bi. It is possible that the BB sons should have been called B?, which has a phenotype between BB and B. If this is correct, we face a parallel to the first case of conversion-like behavior. In the first instance +BB/r+ produced almost 100 per cent gametes B and r B; here +BB/r+ made gametes +B? and r Bi. It should be noticed, also, that only a part of the RF_2 females behaved thus, which again might suggest the presence of a segregating inducing agent; further, that the unequal crossing-over had the tendency to be of the type producing Bi, a tendency which had already been visible in the crossovers in RF_2. Such a tendency must also have a genetic basis, as it was either present or not, while the unequal crossing-over of the type found in the first case was not realized in the present case. Thus the assumed "transductor" would have to produce specifically a break within a Bar segment in this instance. (It should be added that a derivation of the Bi r chromosome, appearing in the F_3 sons, from a crossover chromosome in the F_2 female is not possible because such a female would have to be homozygous rr.)

Further proof of the correctness of the interpretation is derived from additional results. In three other matings of RF_2 females, shown to be +BB/r+, with BB brothers, the sons consisted of *three* classes: $^1/_2$ BB, $^1/_4$ Bi r, and $^1/_4$ r! This is possible either if the unequal crossing-over occurred in only one of two germ track stem cells or, more probably, if this somatic crossing-over occurred between two strand homologues involving only one pair of chromatids, as in genuine synaptic crossing-over. The result would be cells which produce gametes and sons BB, B?, Bi r, r, the first two having been registered as BB.

In favor of this explanation is a test with the females, sisters of the four (distinguishably three) types of males. As the father was BB, sisters looking like BB/BB might be such or B?/BB. Of two crosses of such females with BB males, one bred true for BB/BB. The other had daughters of phenotype BB, but a part had highly different eyes, and sons BB and others looking more like B, i.e., perhaps B?. It is most unfortunate that the latter were not isolated for further tests.

It should be added that a formal explanation for the foregoing facts could also be given, if the unequal crossing-over in the nucleus of the stem cell occurred in the one-strand stage but were dependent upon a dominant, segregating, autosomal modifier. We hope to find some day a repetition of these happenings in a properly marked experiment. As this cannot be promised, we considered a publication of the facts, as they stand, advisable.

Discussion.—The facts presented here, incomplete as they are, seem to be of great importance for the understanding of the strange phenomena which have been so much discussed recently as the "Mary Mitchell effect," "conversion," "trans-replication (work by Renner, Oehlkers, Mitchell, Lindegren, Roman, Laughnan, Brink; discussions by Catcheside, Glass, and others—see the reviews in 1957 *Symposia*[2]). If it were not known that Bar is a duplication of a chromosomal segment with a tendency to unequal crossing-over resulting in a triplication and if the facts were reported in terms of ordinary loci, they would clearly belong to the group of unexpected phenomena described in yeast, *Neurospora*, and maize. But here we know for certain most of what has happened. If a female +BB/r+ forms exclusively gametes (apart from a few crossovers) B and rB, this could be described as a conversion of $+^{B}$ into B in the r-chromosome under the influence of BB. Actually, it is somatic "unequal crossing-over" from a triplication, which raises the question whether the transreplication (Glass) phenomena in plants should not be understood with the facts presented here in mind. A short discussion of the possibilities has been given above for the first case in *Drosophila*, which applies also to a part of the second case. A complete discussion of the cases in plants in the light of the present analysis would be lengthy and should wait until the unfortunate gaps in the present account are filled. A final discussion would involve not only duplications, etc., but the modern ideas on the genetic structure of segments formerly called *genes*.

We pointed to the possibility that the facts might, in principle, be the same as bacterial transduction, especially when somatic chromosomes are involved, which even in Diptera are some distance apart. We know of no mechanical agent of such transduction; but we pointed to the strong hints that a transducing locus is involved here. This again reminds one of McClintock's work, where a "transduced" Ds locus in the presence of Ac (the transductor in the parallel) is responsible for unusual phenomena. If we succeed in filling gaps relating to this "transductor" which seemed to be separable by crossing-over, but was not isolated, it might be possible to bring into line all four groups of facts: transduction, McClintock effect, Mitchell effect, and unequal crossing-over.

Great hopes are attached to the findings about the origin of infrabar, always considered to be a genuine mutant within a Bar section. The facts presented leave no doubt that Bi originates with a transreplication, and it is most probable that in our case the effect is due to a wrong subdivision of the triplicated double Bar

segments at the time of transduction. The final proof would require the isolation of the reciprocal chromosome, which was probably seen but, unfortunately, not isolated, and analysis of the reciprocal salivaries.

Summary.—Two cases are described in *Drosophila* which could be interpreted as conversion. But in these cases the Bar duplication and triplication are involved, which permits an intrepretation in terms of unequal crossing-over, which, however, occurs in all gametes, not merely a few crossover gametes. The happenings are under control of another locus, which in one case can be separated from Bar by crossing-over. The details can be understood best by assuming a transduction of a Bar segment under control of a transductor locus. A final proof by isolating this locus was missed. In the second case, the same phenomenon was combined with the production of infrabar and what looked like its reciprocal, if a break within a Bar segment was involved in the transduction (unequal crossing-over). The final proof for this interpretation is still missing.

[1] R. B. Goldschmidt, *Proc. Nat. Acad. Washington*, **43**, 731–736, 1957.

[2] *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 1957, *Symposium on the Chemical Basis of Heredity* (Baltimore: Johns Hopkins University Press, 1957).

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